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NOTICE

We are happy to announce that beginning with the January, 1962 issue, the American Institute of Biological Sciences, Washington, D.C., will take over a number of functions in connection with publication of this Journal, including redactory services. Circulation and most other business matters have already been transferred to their management, and mechanisms for handling of subscriptions have been revised and simplified.

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Review

The metabolism of adipose tissue *in vitro*

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Although the vital role of adipose tissue as a reservoir of fat which can be mobilized to provide energy has long been recognized, it was assumed until relatively recently that the tissue is to a large extent metabolically inert. By 1948 there was available a considerable body of data derived from physiological studies which indicated that adipose tissue is not an inactive storehouse. This view was stressed by Wertheimer and Shapiro (1) in their classic review, despite absence of precise information concerning the enzymes and metabolic pathways in the tissue.

An important barrier to the understanding of adipose tissue metabolism and its relationship to the physiology of the intact organism was the lack of knowledge of the mechanism by which fat is mobilized from adipose tissue and transported to other tissues. Favarger (2) had theorized in 1949 that free fatty acids (FFA) might be the form in which fat is released from adipose tissue and transported in the blood. The first clear-cut evidence in support of this hypothesis came from the studies of Gordon (3, 4), who correlated the concentration of free fatty acids in serum with changes in nutritional state and interpreted these data as evidence that fat is released from adipose tissue and transported in the blood as FFA. Similar observations were also reported by Dole (5). These and numerous subsequent studies were made possible by the development of reliable, relatively simple methods for quantification of free fatty acids in serum and other biological media by Gordon (4) and by Dole (5). The demonstration that fatty acid release and other metabolic processes are influenced by hormones during incubation *in vitro* has led to widespread interest in the activities of adipose tissue.

There are obvious differences in the metabolism of adipose tissue at different locations in the body. Certain depots are depleted more rapidly during starvation and, conversely, certain areas participate little if at all in the development of generalized obesity. To what extent these differences are due to nervous regulation or to variations in vascularity or control of blood supply is not known. The former is undoubtedly of basic importance in the regulation of adipose tissue metabolism in the intact animal. When tissues are studied *in vitro*, these factors are obviously inoperative except in so far as they have affected the composition (chemical and/or enzymatic) of the particular sample of tissue prior to its excision for study.

Several investigators have compared specific aspects of the metabolism of rat adipose tissue derived from different anatomical sites.[1] In studies of fatty acid synthesis Shapiro and Wertheimer (6) found equivalent activity in mesentery and in groin fat. Minced preparations of epididymal, perirenal, and subcutaneous adipose tissue incorporated roughly equal amounts of glucose carbon into fatty acids (7). Glucose uptake and the effects of insulin on this process were of the same magnitude in tissues from subcutaneous, genital, mesenteric, and perinephric regions (8). Using a manometric method, Ball and Cooper (9) found that the responses of epididymal, mesenteric, and perinephric tissues to insulin were quantitatively alike. Adipose tissues from these same sites obtained from female rats (substituting inguinal fat for epididymal) had equivalent activities and were not grossly different from the tissues of male rats in their response to insulin. It has

[1] In all of these statements and in the discussion that follows, "adipose tissue" refers to white (or yellow) adipose tissue. Brown adipose tissue is distinctly different in location, morphology, composition, etc. A few metabolic differences are reported in the studies comparing adipose tissue from various locations (6, 7, 9). In all studies, with exceptions noted in the text, rat tissue was used. Studies of the metabolism *in vitro* of adipose tissue from animals other than the rat are so few that there is no basis for discussion of species differences.

been found, however, that fatty acid synthesis from glucose and the oxidation of carbon-1 of glucose is greater in adipose tissue from female than from male rats (10).

The information to be summarized here pertains chiefly to the metabolism of the epididymal fat pad which is a particularly convenient source of adipose tissue for study *in vitro*. There is no reason to believe that its metabolism *in vitro* differs significantly from other adipose tissue of the male rat. On the other hand, it is reasonable to assume that the metabolism of adipose tissue from female rats will differ in some particulars.

In this survey, which is based largely on *in vitro* observations, no explicit attempt has been made to assess the physiological significance of the findings. As the available information is summarized and interpreted, however, certain correlations and questions will emerge concerning adipose tissue metabolism and its regulation in the intact animal.

Fatty Acid Metabolism

Reactions leading to the accumulation or removal of FFA in the adipose tissue cell are shown schematically in Figure 1. Release of FFA *in vitro* is readily demonstrable. Although transfer of FFA into the cell can be observed in *in vitro* studies, there is no evidence that

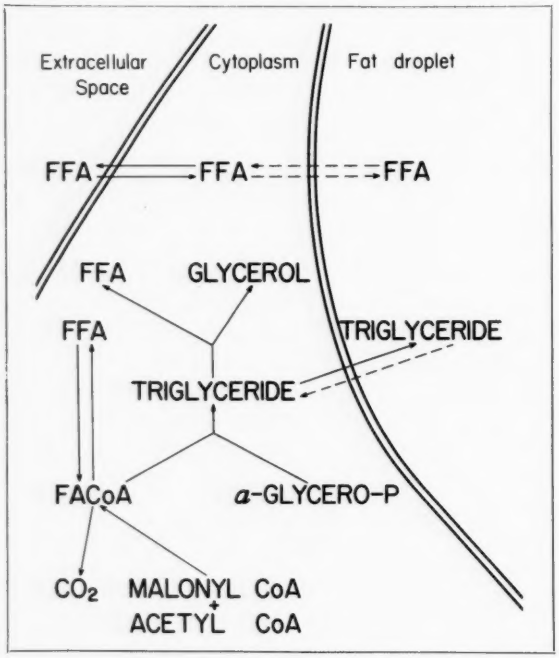

Fig. 1. Production and removal of free fatty acids in adipose tissue.

net uptake of serum FFA by adipose tissue occurs *in vivo*. It is probable that under most conditions *in vivo*, when there is net movement it is in the outward direction.

Fatty acid synthesis is an important function of adipose tissue *in vivo*. The rates of fatty acid synthesis and degradation observed during incubation *in vitro* suggest, however, that these reactions are not involved in the regulation of FFA accumulation and release. It is pertinent in this regard to note that when fatty acid synthesis is most active, fatty acid esterification is also active, and fatty acid release is minimal. Conversely, fatty acid synthesis (and the rate of esterification) is minimal under conditions where FFA release is highest.

Adipose tissue has a large store of triglyceride which, when acted upon by one or another of the lipases found in this tissue, provides the major source of FFA for release. There is considerable evidence to support the view that triglyceride breakdown and resynthesis go on constantly. It is obvious, then, that regulation of the rate of FFA accumulation or release could be effected by altering the rate of lipolysis and/or the rate of esterification. Both of these mechanisms are apparently utilized. Increased FFA release caused by certain hormones is probably associated with increased lipolysis. The suppression of FFA accumulation resulting from the availability of glucose for metabolism likely results from an increase in the rate of esterification of FFA. In this regard it is important to remember that the products of lipolysis, FFA and glycerol, are not the immediate substrates for the esterification reactions. The FFA must first be activated to form fatty acyl coenzyme A derivatives, which in turn react with α-glycerophosphate, not glycerol, in the first steps of glyceride synthesis. Glycerol cannot be utilized to any significant extent by adipose tissue. Thus, the glycerol produced by lipolysis accumulates, providing an index of the amount of triglyceride breakdown; and re-esterification of the fatty acids constantly produced by lipolysis depends on a continuing supply of glucose (or other equivalent substrate) to provide α-glycerophosphate.

The dotted arrows in Figure 1 indicate questions concerning FFA pools and reactions within the adipose tissue cell. The FFA involved in different reactions are indicated separately to suggest that although the tissue fatty acids are frequently referred to as an entity, there is good evidence for the presence of more than one kinetically distinguishable pool. It should not be inferred, however, that each of the "FFA's" on the diagram represents a specific discrete pool. Furthermore, although it is almost certain that at least

a portion of the tissue FFA is dissolved in the lipid phase of the fat droplet, it is not known how this is related functionally to one or another of the metabolic pools of FFA.

It is generally accepted that triglyceride is synthesized in the cytoplasm of the adipose cell and then transferred into the fat droplet where it is stored. It is not known whether triglyceride is transported back to the cytoplasm for hydrolysis or whether there is lipolytic activity at the surface of the fat droplet, so that intact triglycerides do not leave the fat droplet. Movement of triglycerides across the cell membrane is not included in the diagram but some data relating to this question are discussed below.

Figure 1 offers a highly schematic picture of the adipose tissue cell. It, however, serves to emphasize the reactions which are the basis of the most obvious metabolic activities of this tissue.

FATTY ACID METABOLISM IN INTACT TISSUE

Fatty Acid Release. Data from a number of studies of the release of fatty acids by adipose tissue have been reviewed by Engel and White (11) and by Wertheimer and colleagues (12, 13). Some general observations about FFA release are summarized here as a background for discussion of specific information in subsequent sections. The mechanisms through which the effects of hormones on FFA release and on other aspects of metabolism are produced and interrelated are considered below.

In blood, FFA are almost completely bound to albumin (14). In order to demonstrate FFA release from adipose tissue *in vitro*, albumin must be present in the medium (15). The interaction of FFA with albumin has been studied in detail by Goodman (14), who interpreted his data in terms of three classes of binding sites for FFA on the albumin molecule; one group consisting of two sites with a very high association constant, another group of five sites with a somewhat lower constant, and a third group consisting of a much larger number of sites (of the order of twenty) with a much lower association constant. The concentration of unbound FFA, i.e., the chemical potential of FFA, in solutions containing albumin is a function of the mole ratio of fatty acids to albumin. From the mole ratio using the apparent association constants for the binding reactions it is possible to estimate the concentration of unbound FFA.

The chemical potential of FFA in the tissue cannot be approximated in any similar way. Concentrations of FFA in the adipose tissue range from 1 to 2 μmoles per gram in the fed state and 2 to 3 μmoles per gram in fasting, up to 8 to 10 or more μmoles per gram in tissues that have been incubated with epinephrine (16, 17, 18). A fraction of the total FFA is probably bound to protein in the cytoplasm. Although the quantity and properties of the binding protein are unknown, the amount of FFA in this location must be presumed to be small since the total protein of adipose tissue is low. A large portion of the tissue FFA must be contained in the fat droplet which, as an organic phase, extracts FFA from the cytoplasm (and perhaps also directly from the extracellular space).[2] After homogenization of the tissue, a large fraction of the FFA is found in the fat layer separated by centrifugation. The extent to which this is a reflection of the distribution of FFA in the intact cell is not known and is difficult to determine experimentally. Knowledge of the distribution and state of the FFA within the adipose tissue will be required, however, in order to understand their effects on cellular metabolism.

The amount of FFA in the tissue at any time is the dynamic resultant of metabolic factors that influence the quantity of FFA added to and removed from the cell. If movement of FFA in and out of the cell is not an active process, then in an *in vitro* system FFA should be distributed between medium and tissue in such a way that the chemical potential is the same in both compartments. Thus, the net movement of fatty acids will be determined by the chemical potential of fatty acids in the tissue relative to that in the medium. The effect of the concentration of FFA in the medium on net movement is easily demonstrated (15, 19). At low concentrations of FFA in the medium there is net release which ceases as the concentration is increased. With further increases in medium FFA concentration there is net uptake of FFA by the tissue. By keeping the amount of FFA in the medium constant and altering the amount of albumin, it would also be possible to influence the movement of FFA.

The quantitative relationship between concentration of FFA in the medium and net movement is a function of tissue metabolism. There is net release of FFA from adipose tissue of fasted rats into medium containing added FFA (20). Tissues from fed rats, however, release little FFA even into medium containing none (20). In the presence of epinephrine, output of fatty acids continues despite medium concentrations much higher than those which reverse net movement in the absence of the hormone. These differences reflect differences in effective FFA concentration (chemical potential of

[2] The cytoplasm is accumulated in one area of the adipose tissue cell. Over the surface of most of the fat droplet there is no detectable layer of cytoplasm.

fatty acids) in the tissue, maintained by cell metabolism.

It is obvious that the chemical potential of FFA in neither compartment is constant during incubation of tissues *in vitro*. It has been found that under certain conditions the accumulation of FFA in the medium tends to stop after about 1 hour of incubation (21, 22). When incubation is continued for a total of 3 hours, some of the FFA initially released may be taken up again by the tissue. If, on the other hand, the tissue is transferred at the end of 1 hour to fresh medium (containing no FFA), release of FFA is continued at the initial rate for another hour. In addition to indicating again the role of the medium in the release process, these findings suggest that metabolic adjustments occur in association with changes in FFA movement. This is borne out by data from other studies. It has been observed that during the first 30 minutes of incubation of epididymal fat pads from fasted rats, the amount of FFA that appears in the medium is roughly equivalent to the decrease in tissue FFA content that occurs at the same time, i.e., there is no net production of FFA in this time. At the end of 60 minutes of incubation, more FFA is accumulated in the medium and the concentration of FFA in the tissue is restored to the zero time level, i.e., there is net production of FFA during the second 30 minutes of incubation.[3] Metabolic changes associated with fatty acid movement can be demonstrated also when tissues incubated with and without albumin in the medium are compared. The concentration of FFA is higher in tissues incubated in the absence of albumin. The amount of extra FFA in those tissues is, however, less than the amount of FFA found in the medium containing albumin. This suggests that the elevation of FFA concentration in the tissue without albumin retards further accumulation.[3]

Relatively minor differences in experimental conditions, e.g., in the amount of or the FFA content of the albumin used, or the time of incubation, can produce effects on cell metabolism. These findings lend support to the view that certain of the effects of epinephrine and other hormones with similar action are the result of the elevation of intracellular FFA concentration which they produce (23, 24). The results of studies carried out in adipose tissue homogenates or cell fractions also may be influenced by the concentration of FFA in the tissues from which they were prepared. This can be an important variable whether or not incubation of tissues has been carried out prior to homogenization.

As used in this discussion, FFA refers collectively to the several long-chain fatty acids found in adipose tissue. The fatty acids comprising this mixture differ in solubility and other physicochemical properties. Goodman (14) has observed that there is considerable difference in the association constants for the interaction of albumin with different fatty acids. It is to be expected that there are differences in the physiological behavior of the specific fatty acids. Preliminary observations of Watson and Margolis[4] indicate that the fatty acid composition of FFA released from adipose tissue during incubation differs significantly from that of the glyceride fatty acids of the tissue.

Uptake and Utilization of Fatty Acids. Stern and Shapiro (25) first demonstrated the uptake and esterification of stearic acid-1-C^{14} by adipose tissue incubated *in vitro*. Shapiro and co-workers (26, 27, 28) extended these observations and reported that the nutritional state of animals from which tissues were obtained markedly affected the quantity of C^{14}-FFA taken up and esterified *in vitro*. In general, more than 90% of the C^{14}-FFA taken up by tissues from fed rats is found in the ester (triglyceride) fraction at the end of the incubation. Tissues from fasted rats take up less C^{14}-FFA and of the amount contained in the tissue, less than 70% is in the glyceride fraction[5] (27). The uptake and esterification of C^{14}-FFA by tissues from fasted rats takes place simultaneously with a net release of FFA, whereas the tissues from fed rats release little or no FFA.

Incubation in nitrogen inhibits incorporation of palmitic acid-1-C^{14} into glycerides by only 60%. The addition of 0.01 M sodium fluoride to tissues incubated in nitrogen causes little more inhibition, although in the presence of both 0.1 M sodium fluoride and nitrogen, incorporation of C^{14}-palmitate was almost completely abolished. It is interesting that there is little or no increase in FFA release associated with inhibition of C^{14}-palmitate incorporation by nitrogen and/or fluoride. This means either that fatty acid production (lipolysis) is inhibited along with esterification or that the rate of breakdown and resynthesis of triglycerides is so slow that what appears to be almost complete inhibition of esterification does not lead to a sizable accumulation of FFA during the incubation period. Similarly, apparently large effects of glucose on FFA esterification are associated with relatively small net changes in FFA release (29).

The incorporation of palmitic acid-1-C^{14} into tissue glycerides is increased by small amounts of glucose (0.625 μmole/ml) (19). When glucose (or glucose plus insulin) is added to the medium, the rate of FFA release is decreased and there may be, in fact, a net disap-

[3] M. Vaughan and D. Steinberg, unpublished observations.

[4] W. C. Watson and S. Margolis, unpublished observations.

[5] D. Steinberg, M. Vaughan, and S. Margolis, unpublished observations.

pearance of FFA (20, 30, 31). The finding that glycerol release continues when FFA release is decreased by glucose (32) suggests that lipolysis continues unchanged, leading to the formation of FFA and glycerol, but that in the presence of glucose the rate of triglyceride synthesis is enhanced so that little or no FFA accumulates.

When the concentration of fatty acids in the medium is increased, an increase in net uptake of fatty acids can be observed (see above). The extra FFA removed from the medium is not accumulated in the tissue and this, together with data on the incorporation of palmitic acid-1-C^{14} into triglyceride in similar experiments (19), suggests that the rate of fatty acid esterification is increased.

It has been found that about 3% of the C^{14}-palmitate present in the tissue at the end of a 1-hour incubation period is in the phospholipid fraction and is recovered in one component after chromatography of this fraction on silicic-acid paper (33). The percentage of tissue radioactivity in this fraction was the same in tissues from fed as from fasted rats, i.e., there was more radioactive palmitate in the phospholipid fraction in the tissues from fed rats. The addition of glucose to the medium had considerably less effect on incorporation of palmitate into phospholipid than it did on its incorporation into triglyceride determined in the same tissue.[6] If glucose stimulates triglyceride synthesis by acting as a precursor for α-glycerophosphate and/or a source of energy for fatty acid activation, then it must be concluded that neither the availability of α-glycerophosphate nor the rate of fatty acid activation is the factor limiting incorporation of palmitate into phospholipid in these experiments.

In the case of the glucose effect, all available evidence supports the interpretation that the increased incorporation of C^{14}-palmitate into neutral lipid results from an increase in the rate of triglyceride synthesis. It is not possible, however, to deduce the true rate of fatty acid esterification in experiments such as those described above since the specific activity of the fatty acids of the precursor pool is not known. From the previous discussion of the state of FFA in adipose tissue, it seems clear that the total FFA of tissue is not the precursor pool. Circumstantial evidence in support of this conclusion is obtained from attempts to calculate a minimum value for the amount of fatty acid esterified, using the specific activity of the tissue FFA at the end of the experiment. (The specific activity of the tissue FFA is zero at the beginning of the experiment, rises relatively slowly, and even at the end of a 3-hour incubation period is only a very small fraction of the specific activity of the medium fatty acid [29, 34]). In many instances the rates of esterification calculated in this way are implausibly high, suggesting that the specific activity of the true precursor pool is much higher than that of the total tissue fatty acids.

Kerpel *et al.* (34) have attempted to assess the extent of participation of the tissue FFA in the incorporation of C^{14}-palmitate from the medium into tissue glycerides and in the formation of medium fatty acids from tissue glycerides. They have concluded that "the tissue free fatty acids do not seem to take a prominent part in assimilation and esterification." In addition, they have presented evidence for the presence of a triglyceride compartment associated with a particulate fraction which had a much higher specific activity than the triglyceride of the floating fat, and have suggested that this "active" glyceride compartment interacts directly with the medium fatty acids without the participation of the tissue FFA. There seems to be agreement on the fact that the total adipose tissue FFA is not the precursor pool for glyceride synthesis, but it is quite possible that the tissue contains a pool that is the precursor.

In an attempt to demonstrate the existence within the adipose tissue cell of fractions containing fatty acids of different specific activity, homogenates of tissues incubated for short periods with C^{14}-palmitate were separated into three fractions by centrifugation.[6] Table 1 contains representative data from a few such studies, showing distinct differences in the specific activity of fatty acids of cell fractions separated in this crude fashion. It is not presumed that any of these fractions represents a single metabolic pool or a specific combination of metabolic pools. No one of them has a

TABLE 1. SPECIFIC ACTIVITY OF FATTY ACIDS IN CELL FRACTIONS AFTER INCUBATION OF ADIPOSE TISSUE WITH PALMITIC ACID-1-C^{14}

Exp. No.	FFA Specific Activity (cpm/μEq FFA)			
	Whole Homogenate	Sediment	Solution	Fat
1	1980	3050	390	921
2	1530	4740	730	1060
3	1260	2690	692	869
4*	800	3420		590
5*	1110	3860	700	739

Both fat pads from each rat (fasted) incubated together for 20 minutes in medium containing 0.25 or 0.5 μc of palmitic acid-1-C^{14}. Fractions separated by centrifugation of homogenized tissue for 30 minutes at 15,000 $\times$ g, at 4°.

* Medium in which tissues were incubated contained epinephrine 0.1μg/ml.

[6] M. Vaughan, unpublished observations.

specific activity which would be expected for the pool that is the precursor of triglyceride. The composition of these fractions is not known; certainly all are heterogeneous. Even if there are functionally and kinetically distinguishable pools, the possibility of isolating one or another is questionable, since the latter achievement depends on the association (maintained throughout the isolation procedure) of a specific pool with a morphologically distinguishable and isolatable cell fraction. Although the fatty acids in the sediment have the highest specific activity, it is possible that one or another of the fractions includes a very small pool of very high specific activity.

Table 2 contains data of another type, indicating a practical problem and a potential artifact in many kinds of experiments with intact tissues.[6] Intact epididymal fat pads were incubated with palmitic acid-1-C^{14}, then divided into two pieces, a thick (proximal) portion and a thin (distal) portion. Each piece was homogenized separately and the glyceride and fatty acid fractions isolated. The explanation for the differences in fatty acid specific activity in different portions of the same tissue is not readily apparent, but the generally greater accumulation of C^{14} in glycerides of the thin portion, when expressed per gram of tissue, strongly suggests that there may be areas of the thick portion that participate to a variable extent in the esterification of the labeled fatty acid from the medium. In the presence of gradients within the tissue, e.g., only a portion of the cells participating in fatty acid uptake and release, the specific activity of the total tissue fatty acid is not a relevant datum.

If the interpretation of Kerpel *et al.* (34) were correct, i.e., if there were "direct interchange between medium fatty acids and tissue fatty acid esters," it would be possible to approximate (estimate) rates of fatty acid esterification, although the calculation would be complicated, however, by the fact that the specific activity of the medium FFA decreases throughout the incubation. In experiments where there is uptake of labeled fatty acid (tracer amount) associated with net release of FFA, the specific activity of the medium FFA may decline to 25% of its initial value in 1 hour.[3] Changes in medium specific activity have been amply documented by Kerpel *et al.* (34), who observed that even when there was no net release of fatty acids (tissues from fed rats incubated in medium containing glucose), the specific activity of the medium fatty acids fell to less than 40% of the zero time value after 3 hours. Assuming that the medium FFA is very nearly in equilibrium with the precursor pool, it is apparent that calculations of esterification based on the initial medium specific activity would be of questionable usefulness, especially when attempting to compare rates of synthesis in two tissues where there have been gross differences in the change of specific activity of medium fatty acids with time.

At present it seems probable that there is in adipose tissue a pool of fatty acids that is the precursor for triglyceride. It is most likely small and must be of much higher specific activity than the total tissue FFA. Perhaps it is very nearly in equilibrium with the medium fatty acids. The presence of another pool of tissue FFA comprising FFA from triglyceride breakdown in the process of release may also be postulated. The relationship of FFA in the fat droplet to these or other possible metabolic pools remains unknown.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY FROM C^{14}-PALMITATE WITHIN THE EPIDIDYMAL FAT PAD

Tissue No.		Weight	Tissue FFA		Tissue Glycerides
		mg.	*μeq/g*	*cpm/μeq*	*cpm/g*
1	Thick	305	1.8	2,060	3,930
	Thin	370	2.0	1,260	6,510
2	Thick	290	2.2		2,110
	Thin	408	1.9	1,575	7,750
3	Thick	272	1.8	2,120	3,220
	Thin	445	1.7	1,450	3,820
4	Thick	318	1.9	1,950	3,640
	Thin	383	1.6	1,400	3,510
5*	Thick	250	1.4	7,410	38,800
	Thin	256	1.8	1,250	66,900
6*	Thick	359	1.3	2,280	31,100
	Thin	195	1.9	2,790	42,600
7*	Thick	200	1.5	3,950	39,200
	Thin	574	1.1	3,010	57,900
8*	Thick	297	1.2	7,690	
	Thin	475	1.3	1,150	

Each fat pad (intact) was incubated for about 30 minutes at 37° in 3 ml medium containing 0.25μc palmitic acid-1-C^{14}. It was then rinsed, blotted, and divided into a thick (proximal) and thin (distal) portion, each of which was then weighed, homogenized, and the lipids extracted and fractionated.

* In these experiments the albumin in the medium had been extracted to free it of FFA, i.e., the specific activity of medium FFA was much higher than it was in Nos. 1–4.

Fatty Acid Synthesis and Oxidation. The synthesis of fatty acids from various isotopically labeled precursors in intact adipose tissue has been extensively studied. Winegrad *et al.* (35) considered in detail much of the available data on lipogenesis in adipose tissue. Only a brief summary is presented here. Some of the pertinent findings will be mentioned later in considering the effects of hormones, several of which

have been shown to influence fatty acid synthesis. Shapiro and Wertheimer (6) first demonstrated the synthesis of fatty acids in adipose tissue by measuring the incorporation of deuterium from deuterium oxide. Fatty acid synthesis from C^{14}-labeled acetate, pyruvate, malonate, lactate, acetaldehyde, propionate, and isoleucine has been studied in a number of laboratories (21, 36 to 45). Utilization of glucose for fatty acid synthesis is discussed along with other aspects of glucose metabolism (see below).

Lipogenesis is markedly depressed in adipose tissue from starved rats and also in tissue from rats that have been maintained on a high fat, low carbohydrate diet (7, 39). Fatty acid synthesis from acetate is essentially zero in tissues from starved rats and is only partially restored by the addition of glucose to the medium (39). Furthermore, glucose uptake is low and fatty acid synthesis from glucose itself is diminished in tissues from fasted rats (39, 46). Presumably the effect of fasting on lipogenesis is a manifestation of the close relationship between the rate of fatty acid synthesis and the amount of glucose metabolized through the oxidative pathway. The correlation between lipogenesis and activity of the phosphogluconate shunt has been observed in other tissues under a variety of conditions. As noted earlier, adipose tissue from female rats, which oxidizes carbon-1 of glucose more rapidly than does tissue from male rats, also exhibits a greater rate of lipogenesis (10). When the amount of glucose metabolized through the oxidative pathway is increased by insulin or by increasing medium glucose concentration, fatty acid synthesis is stimulated (43, 47, 48). On the other hand, under conditions in which glucose uptake is low, or when glucose utilization, though high, proceeds to only a limited extent through the phosphogluconate shunt (e.g., in the presence of epinephrine), fatty acid synthesis is depressed (23, 49). It is not known through what mechanism this relationship is maintained. A number of workers have considered that the rate of fatty acid synthesis is in some way dependent upon, or regulated by, the availability of TPNH generated in the oxidation of glucose-6-phosphate. Leboeuf and Cahill (50) have suggested, alternatively, that the availability of acetyl-CoA for fatty acid synthesis influences the pathway taken by glucose.

It may be noted that although liver and adipose tissue respond to fasting similarly with a marked depression of fatty acid synthesis, the two tissues differ in their response to other variables. Thus, exposure of rats to cold, which abolishes hepatic lipogenesis (51), has very little effect on lipogenesis in adipose tissue (52). The latter is also unaffected by the addition of ethanol, which stimulates fatty acid synthesis from acetate in liver slices. (The absence of an effect of ethanol in adipose tissue may be related to the fact that ethanol-1,2-C^{14} is apparently not oxidized in this tissue [53].) The addition of insulin *in vitro* does not restore fatty acid synthesis in slices of liver from diabetic rats (54), whereas the depression of fatty acid synthesis in the adipose tissue can be relieved by addition of insulin to the incubation medium (35, 43).

Oxidation of C^{14}-labeled fatty acid by adipose tissue *in vitro* has been observed in several laboratories (19, 21, 26, 27, 55). The amount of fatty acid oxidized is not large and the formation of $C^{14}O_2$ from labeled fatty acid is depressed by the addition of small amounts of glucose to the medium (19). Shapiro and co-workers (26, 27) found that the fraction of labeled fatty acid taken up by the tissue that was converted to carbon dioxide was larger in tissues from fasted rats than in tissues from fed rats. The actual amount oxidized was, however, approximately equal in both types of tissues. Production of $C^{14}O_2$ from palmitic acid-1-C^{14} was roughly proportional to the total concentration of fatty acids in the medium (19). For reasons discussed above, however, it is difficult to calculate in such experiments the actual rates of fatty acid oxidation.

Uptake and Release of Triglycerides. It is generally agreed that adipose tissue *in vivo* derives its supply of preformed fatty acids from the triglycerides of low density lipoproteins. A role for lipoprotein lipase in this process has been postulated. It has been suggested that lipoprotein lipase situated on the endothelium of capillaries in adipose tissue (or in some similar position near the circulating blood) hydrolyzes triglycerides of the lipoprotein, yielding FFA and perhaps partial glycerides, which then readily pass from the vascular space into the adipose tissue cells (56).

Such a mechanism would likely not be observable in *in vitro* studies, since under these conditions the substrate is not delivered to the adipose cells through the capillary circulation. Thus, Rodbell (57) concluded on the basis of experiments in which epididymal fat pads were incubated in medium containing chylomicrons or a synthetic triglyceride emulsion that triglyceride uptake might be occurring by pinocytosis. Addition of diisopropyl fluorophosphate, an inhibitor of lipoprotein lipase, to the medium did not interfere with uptake. Furthermore, the triglycerides were apparently taken up intact without prior hydrolysis. Following uptake by the tissue into a compartment no longer exchangeable with triglycerides in the medium, the triglycerides were apparently hydrolyzed and fatty acids re-esterified prior to storage in the fat droplet. In tissues from fasted rats the process of hydrolysis and re-esterification was

less active than it was in tissues from fed rats, although the uptake of triglycerides was the same in the two groups.

It has been generally assumed that triglycerides are not released from the adipose tissue cell. Just as the release of fatty acids requires the presence of albumin, triglyceride release into an aqueous medium is not likely to occur in the absence of a suitable acceptor. The nature of the "suitable acceptor," if one exists, is unknown, but lipoprotein comes to mind as a reasonable possibility and it has been reported by Shapiro (28) that release of triglyceride can be demonstrated when β-lipoprotein is included in the incubation medium. This process has not been further characterized and, like the problem of triglyceride uptake, offers many questions for solution.

ENZYMATIC PATHWAYS OF FATTY ACID METABOLISM

Fatty Acid Synthesis. Wakil (58) has recently summarized in detail the information concerning the mechanisms of fatty acid synthesis in living organisms. The mitochondrial system which seems to be concerned chiefly with the addition of two carbon units to existing fatty acids has not been studied in adipose tissue. Ganguly (59) studied the distribution of the nonmitochondrial or malonyl-CoA system in various animal tissues. In a comparison of beef tissues, when activity was expressed per milligram of tissue protein, the tissues with highest activity were suprarenal fat, mammary gland, and adipose tissue. Brain was the only other tissue with activity in a similar range. In the past year Martin *et al.* (60) have described the partial purification of this enzyme system from the supernatant solution obtained by centrifugation of homogenates of rat epididymal fat pad for 60 minutes at 105,000 $\times$ *g*. They have determined that when malonyl-CoA, acetyl-CoA, and TPNH are incubated with the enzyme, palmitic acid is the principal product. The ratio of malonyl-CoA to acetyl-CoA utilized for palmitic acid synthesis is 7:1, indicating that the over-all reaction is:

$$7 \text{ malonyl-COA} + \text{acetyl-CoA} + 14 \text{ TPNH} \rightarrow \text{palmitate} + 14 \text{ TPN} + 7 \text{ CO}_2 + 8 \text{ CoA}.$$

It has been suggested that the acetyl moiety from acetyl-CoA forms only the methyl terminus of the palmitate molecule. The rest of the molecule is formed stepwise with two carbons derived from each malonyl-CoA. Consonant with this interpretation, it has also been shown that when a branched-chain or an odd-numbered fatty acyl-CoA derivative is substituted for acetyl-CoA, the corresponding long-chain fatty acid is formed with the acetyl-CoA substitute in the methyl terminal position (61). It is of interest that in all instances, whatever the nature of the acyl-CoA acceptor, the fatty acid produced contained 15, 16, or 17 carbon atoms. It differs in this regard from the yeast enzyme system described by Lynen (62), which yields chiefly fatty acids of 18 carbons in length. Presumably, the palmitic acid and any other fatty acids that are synthesized by this system can be elongated by the mitochondrial system.

There is no evidence available concerning the presence or absence of a soluble system for synthesis of short-chain fatty acids analogous to that described in mammary gland by Hele *et al.* (63). The older literature contains a few reports of enzymes that desaturate fatty acids (64 to 67), but the mechanism by which double bonds are introduced into fatty acids in animal tissues is still under investigation and the system in adipose tissue has not been characterized.

Fatty Acid Esterification: Glyceride Synthesis. The synthesis of glycerides by homogenized adipose tissue has been investigated in detail by Steinberg and coworkers (68, 69). Their observations are compatible with a pathway similar in all respects to that previously described in liver homogenates (70 to 73).

(1) Palmitate + ATP $\rightarrow$ palmityl-CoA + AMP + PP
(2) 2 Palmityl-CoA + α-glycerophosphate $\rightarrow$ phosphatidic acid
(3) Phosphatidic acid $\rightarrow$ diglyceride + phosphate
(4) Diglyceride + palmityl-CoA $\rightarrow$ triglyceride

Table 3 indicates the composition of the system used in studies of triglyceride synthesis in adipose tissue homogenates and the effects of omission of one or another of the components. All of the enzymes required for the synthesis of triglyceride are found in a particulate fraction of the adipose tissue homogenate prepared by centrifugation at 105,000 $\times$ *g* for 1 hour. Active particles can be obtained after suspension and resedimentation of this fraction, although such preparations rapidly lose activity if stored in dilute suspension for a few hours in the cold.

In studying some of these reactions in adipose tissue microsomes, it has been possible to substitute palmityl-CoA for ATP and CoA[7] (74). In this and other homogenate fractions, however, the utilization of acyl-CoA is complicated by the presence of acyl-CoA deacylase activity.[8] Shapiro and co-workers (26), using the hydroxamate method, demonstrated the presence in extracts of adipose tissue of a long-chain fatty acid activating system dependent on the addition of ATP

[7] S. Margolis and M. Vaughan, unpublished observations.
[8] S. Margolis, unpublished observations.

and CoA. This is perhaps the same enzyme that catalyzes reaction 1 in the system described in Table 3 and has been referred to as fatty acyl-CoA synthetase (75) or acyl-CoA thiokinase (76).

In the absence of added α-glycerophosphate there is always some palmitate esterified, even in dialyzed homogenates. This may be due to the presence of small amounts of α-glycerophosphate in the enzyme preparation. It has been found that dialysis under conditions used in preparation for the incorporation studies removes only two-thirds of a tracer amount of α-glycerophosphate-1-C^{14} added to the homogenate. Adipose tissue contains 0.1 to 0.2 μmole α-glycerophosphate per gram tissue (wet weight).[8] Diglycerides, present or formed in the homogenate, also may serve as acceptors for the small amount of fatty acid esterified in the absence of added α-glycerophosphate.

TABLE 3. INCORPORATION OF PALMITIC ACID-1-C^{14} INTO NEUTRAL LIPID BY ADIPOSE TISSUE HOMOGENATE

Additions	Relative Count Incorporation	
	Whole Homogenate	Defatted Homogenate
Complete system*	100	100
Complete system minus ATP	0	1
Complete system minus CoA	4	7
Complete system minus αGP	27	17
Complete system minus Mg^{++}	35	57
Complete system minus cysteine	51	
Complete system minus NaF	32	56
Complete system minus buffer	0	
Complete system with Tris buffer in place of phosphate		93
Complete system minus creatine phosphate	58	
Complete system minus ATP plus 2 μmoles of ADP and 7.5 μmoles of creatine phosphate		45
None	0	
Buffer only	2	
Complete system, held at 100° for 5 minutes	0	

* Each flask contained 2 ml of a 1:5 homogenate of epididymal fat pad in 0.15 M KCl (representing about 400 mg wet weight of adipose tissue) with 10 μmoles of αGP, tracer amount (less than 0.01 μmole) palmitic acid-1-C^{14}, potassium salt, and the following cofactors: 2 μmoles of ATP; 3 μmoles of $MgCl_2$; 0.1 μmole of CoA; 25 μmoles of cysteine; 125 μmoles of NaF; 125 μmoles of potassium phosphate buffer, pH 7.0; water to make a final volume of 3 ml. In addition, incubation done with the whole homogenate contained 7.5 μmoles of creatine phosphate; incubations with the defatted homogenate contained no creatine phosphate except where indicated. Incubation carried out in 25-ml Erlenmeyer flasks for 20 minutes at 37° with shaking.

Reproduced by permission from (69), Steinberg, D., M. Vaughan and S. Margolis. *J. Biol. Chem.* **236**: 1631, 1961.

Labeled phosphatidic acid has been isolated after addition to the system of either palmitic acid-1-C^{14}, α-glycerophosphate-1-C^{14}, or P^{32}-α-glycerophosphate.[7] Phosphatidic acid is the only labeled phospholipid that is synthesized in detectable amounts from any of these precursors in the system described.

Essentially, all of the phosphatidic acid phosphatase activity (reaction 3) is present in the microsomes. Activity varies little with pH between 6.5 and 8.0, where it is maximal. In contrast to the phosphatidic acid phosphatase of liver (71), the adipose tissue enzyme is not inhibited by $MgCl_2$ at concentrations equimolar with substrate.[7]

It might be convenient to call the enzyme that catalyzes reaction 4 diglyceride transacylase. To do so assumes that this enzyme differs from glycerophosphate acylase (76) (or phosphoglycerol transacylase [75]), the enzyme that catalyzes reaction 2. Although not absolutely proved, it seems likely that this assumption is correct. Goldman and Vagelos (74) have studied this reaction in microsomes using C^{14}-labeled palmityl-CoA and oleyl-CoA and several diglycerides. In general, palmityl-CoA was more reactive than oleyl-CoA, and diglycerides containing myristate were more reactive than those containing palmitate, which were more reactive than those containing stearate. The only α,α′-diglyceride tested was unreactive whereas all α,β-diglycerides tested were utilized. The α,β-diglycerides containing an unsaturated fatty acid in either position were apparently more reactive than those containing two saturated fatty acids.

As might have been expected from the results of studies with C^{14}-labeled glycerol in intact adipose tissue[5] (21, 27, 46), α-glycerophosphate cannot be replaced by equimolar amounts of glycerol (69). When added in very high concentration, glycerol can be esterified in a system containing the CoA ester of palmitic acid-1-C^{14} and either microsomes or a soluble supernatant fraction. Certain other alcohols can also serve as fatty acid acceptors in this system.[8] From data obtained in the studies with glycerol, it was deduced that monoglycerides also can be esterified, although it has not been possible to demonstrate directly the esterification of monoolein. When monoolein (10 μmoles) was substituted for α-glycerophosphate in the system described in Table 1, 40% of it was hydrolyzed during the 30-minute incubation period (69).

No evidence for the formation of α-glycerophosphate from glycerol and ATP (glycerokinase activity) in adipose tissue has been found[8] (77) and it seems clear that glycerol is not an effective precursor for glyceride synthesis at concentrations which for α-glycerophosphate are optimal. In suitably fortified homogenates, any

of the metabolic intermediates between glucose-6-phosphate and α-glycerophosphate can be substituted for the latter in supporting glyceride synthesis (78). (See below.)

In homogenates of adipose tissue in which glyceride synthesis is studied, lipolysis goes on simultaneously with esterification (69). When unfractionated homogenate is incubated at 37° without additions, there is a net increase in FFA in the system. When the homogenate is supplemented for glyceride synthesis, as in Table 3, FFA accumulation is diminished and in some instances there is a net disappearance of FFA. It has been shown that there is essentially no synthesis or oxidation of FFA under these conditions, so that it may be presumed that this difference represents a net synthesis of glycerides. The specific activity of the FFA decreases markedly over the course of such experiments, indicating that lipolysis is continuing even when there is a net decrease in FFA. Table 4 contains data from an experiment with homogenate from which most of the neutral lipid (and some of the FFA) had been removed. In the flasks incubated without added FFA there was no net change in fatty acid but a decrease in the specific activity of the FFA to about 12% of the initial level in 20 minutes. In flasks to which 1.2 μmoles of FFA had been added there was a net disappearance of 0.8 μeq of FFA and no change in specific activity.

TABLE 4. EFFECT OF INCREASING INITIAL FFA CONCENTRATION ON NET CHANGES IN FFA AND PALMITIC ACID-1-C^{14} INCORPORATION*

	FFA Added†	Zero Time	20 Minutes
Total FFA	0	0.4	0.4
(μeq per flask)	+	1.5	0.7
FFA specific radioactivity	0	97,000	12,000
(cpm per μeq)	+	25,000	28,000
Neutral lipid radioactivity	0	200	20,400
(cpm)	+	200	12,900

* Defatted homogenate incubated as described in Table 3, including creatine phosphate.

† 1.2 μeq of FFA prepared by saponification of epididymal fat pad lipids.

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When there are large changes in the specific activity of the FFA during an incubation, it is difficult, if not impossible, to draw conclusions about rates of esterifications. Alterations in the incorporation of radioactivity in neutral lipid could be the result of effects on the precursor specific activity (i.e., changes in rates of lipolysis) rather than effects on esterification. Although this presents a complicating factor in studying glyceride synthesis in certain types of homogenate preparations, it offers a system in which regulation of fatty acid metabolism might be investigated, since esterification and lipolysis seem to be the processes chiefly involved in removal and production of FFA. It is of interest that there is no transesterification, i.e., no incorporation of palmitic acid-1-C^{14} into glycerides in the absence of ATP and/or CoA, associated with lipolysis in this system. Similarly, the data of Shapiro *et al.* (79) suggest that there is no incorporation of palmitic acid-1-C^{14} into glycerides by transesterification in intact mesentery.

Lipases of Adipose Tissue. There are apparently several lipases in adipose tissue. Some of the earliest studies of enzymes in this tissue were those of Quagliariello and Scoz (80), who observed hydrolysis of tributyrin and triolein by extracts of adipose tissue. Lipase (or perhaps better, esterase) activity has also been investigated using various Tweens as substrates (81) Mashburn and co-workers have recently described the presence of three presumably different esterases in extracts of adipose tissue (82). One hydrolyzes naphthol acetate, one naphthol caprylate, and the third catalyzes the hydrolysis of tributyrin. The activity of the last of these enzymes was increased in tissues that were incubated with epinephrine or with ACTH prior to homogenization for assay of lipase activity. The partial purification and some of the properties of a lipase that hydrolyzes a wide range of triglycerides but does not attack triacetin or triolein has also been described by Lynn and Perryman (83). No one of these enzymes has been extensively purified and characterized, nor is it possible to assign a physiological role to any one of them.

Lipoprotein lipase is probably the most studied and best characterized of the adipose tissue lipases, although attempts to purify it have been for the most part unsuccessful (84 to 89). The enzyme catalyzes the hydrolysis of glycerides of long-chain fatty acids when they are in the form of lipoproteins, either chylomicrons, low density serum lipoproteins, or artificial substrates consisting of a triglyceride emulsion mixed with serum lipoproteins. Products of reaction are fatty acids, glycerol, and an uncharacterized lipoprotein moiety. The reaction requires any one of several cations for activation and albumin or Ca^{++} as a fatty acid acceptor (88).

Lipoprotein lipase hydrolyzes all three ester bonds of the triglyceride molecule and apparently has no positional specificity (89). It differs therein from pancreatic lipase, which hydrolyzes preferentially those ester bonds involving the α positions of glycerol (90. 91).

This enzyme is characterized by its sensitivity to a number of inhibitors, e.g., protamine, ethylenediaminetetraacetate, phosphate, and sodium chloride (86, 87). Lipoprotein lipase is inactivated by extracts of bacteria adapted to grow on heparin but not by extracts of unadapted bacteria (86). Extracts of both types of this organism contain a mucopolysaccharase that degrades chondroitin sulfates A and C and hyaluronic acid but only the extracts of the adapted bacteria can degrade heparin. On the basis of these observations Korn has suggested that the enzyme may be a mucoprotein with a heparin-like prosthetic group (86).

The precise location of lipoprotein lipase in adipose tissue is not known. Robinson and French (92) have shown that when heparin is injected into the femoral artery of a rabbit, lipoprotein lipase and heparin appear simultaneously in the blood collected from the cannulated femoral vein. They also have found that lipoprotein lipase appears in the blood after injection of dextran sulfate with a molecular weight of 2 million. On the basis of these observations they have suggested that the enzyme must be located very near the circulating blood, perhaps in the capillary endothelium.

When rat epididymal fat pads are incubated in medium containing heparin, lipoprotein lipase is released into the medium (88, 93). Tissues obtained from fasted animals release much less enzyme than do tissues from fed rats (88, 93). The concentration of lipoprotein lipase is also lower in the tissues from fasted rats (93, 94) and it has been demonstrated that this is not due to the presence of an inhibitor in these tissues (93). Incubation of tissues from fasted rats in medium containing glucose and insulin causes an increase in the activity of the enzyme (93).

The available information suggests that lipoprotein lipase functions in the transport of fat from the blood into the adipose tissue and is not involved in the liberation of free fatty acids from adipose tissue glycerides. The fact that triglyceride in the form of lipoprotein is the preferred substrate, the evidence suggesting a location in the capillary wall, and the data correlating enzyme activity with fat uptake by adipose tissue but not with the rate of fatty acid release, provide support for this view. It has been suggested that FFA formed from triglyceride by the action of lipoprotein lipase move into the extracellular space and then into the adipose tissue cell where they are esterified and stored as triglycerides (56). It is of significance in this regard that fatty acid esterification is most active in adipose tissue from fed rats where lipoprotein lipase activity is also at a maximum.

Rizack (95) has described an "epinephrine-sensitive lipolytic system" in adipose tissue. This lipase is contained in particles that do not sediment with centrifugation for 30 minutes at 105,000 $\times$ *g* but do sediment when centrifugation is continued for 12 hours. It is assayed using diluted Ediol® (a commercial coconut oil emulsion), albumin, and phosphate buffer, pH 6.8. Increasing the concentration of phosphate from 2.4×10^{-3} M to 6×10^{-3} M increases enzyme activity about 2.5-fold. There is no change in activity with higher concentrations. It is not inhibited by phosphate, protamine, sodium chloride, or ethylenediamine-tetraacetate as is liproprotein lipase. Sodium fluoride at concentrations that do not affect lipoprotein lipase activity causes marked inhibition.

Hollenberg and co-workers (96) have studied an enzyme that is very similar to the one investigated by Rizack. The two enzymes are assayed in similar systems, have similar pH optima, decline in activity in tissues incubated *in vitro*, and, most striking, are increased in activity in tissues that have been incubated with epinephrine (95, 96) or ACTH (96) but not with growth hormone (96). The evidence strongly suggests that it is the same enzyme that has been studied by both workers. Robinson (97) also may have been observing this enzyme when, in the course of studies on lipoprotein lipase, he noted that extracts from tissues of starved rats hydrolyzed the triglycerides of adipose tissue but not those of added chyle.

It is of considerable interest that Rizack (95) has observed activation of the lipase when extracts of tissues that had lost activity during incubation were incubated with epinephrine and ATP. Information presently available is insufficient, but it seems likely that further investigation will establish this lipase as the one responsible for the breakdown of adipose tissue triglycerides and perhaps for the effects of certain hormones on the release of FFA.

Carbohydrate Metabolism

Metabolism of Hexoses in Intact Tissue

Metabolism of Glucose in Intact Tissue: Role in Fatty Acid Synthesis and Esterification. Data concerning the fate of C^{14}-labeled glucose in adipose tissue and the pathways of glucose metabolism in relation to fatty acid metabolism have been summarized in detail by Cahill and co-workers (46, 49, 98) and by Winegrad *et al.* (35).

By comparing the incorporation of C^{14} from glucose-1-C^{14} and from glucose-6-C^{14} into CO_2 and lipids, Winegrad and Renold (47) have obtained evidence for the presence in adipose tissue of an active phosphogluconate pathway in addition to the glycolytic pathway for glu-

cose metabolism. It was estimated in their experiments that about 50% of the metabolized glucose traversed the glycolytic pathway and 50% the oxidative or another alternate pathway. Other workers using somewhat different assumptions have arrived at roughly the same estimate (98). Winegrad *et al.* (99) have suggested the presence of an active glucuronic acid pathway in adipose tissue on the basis of data obtained in studies of the effects of growth hormone on the metabolism of specifically labeled glucose. In this regard, it has been shown that C^{14} from glucuronic acid-6-C^{14} is incorporated into CO_2 but not into fatty acids, which is the result to be expected on the basis of the known reactions of the uronic acid pathway (35).

More than 70% of the labeled glucose taken up by adipose tissue is recovered in CO_2, glyceride glycerol, fatty acids, and glycogen, the last representing the quantitatively least significant of these components (43, 46, 48, 98). In the presence of low concentrations of glucose in the medium, a large fraction of the total uptake (30% to 50%) is recovered in glyceride glycerol. As the concentration of glucose is increased, glucose uptake rises and the fraction converted to glycerol decreases (46, 48, 98, 100). When tissues are incubated in an atmosphere of nitrogen instead of oxygen there is a marked decrease in glucose uptake, but the incorporation into glyceride glycerol is diminished to a lesser extent than is incorporation into CO_2, glycogen, or fatty acids (46). Tissues from fasted rats exhibit a depressed rate of glucose uptake. Conversion to fatty acids and glycogen is almost abolished and oxidation is markedly diminished. Incorporation of C^{14} from glucose into glyceride glycerol is also affected but to a much lesser extent (46, 100). In all of these circumstances, then, it appears that fatty acid synthesis is most responsive to the availability of glucose within the tissue, whereas production of α-glycerophosphate, the precursor of glyceride glycerol, is maintained with relatively small alterations in the face of large changes in total glucose utilization. The relationship between glucose utilization and lipogenesis has been observed under numerous conditions in adipose tissue and in other tissues. More specifically, it appears that the rate of fatty acid synthesis is related to the amount of glucose oxidized via the phosphoglyconate shunt (see above).

These generalizations fit readily into the picture of an adipose tissue cell in which triglyceride breakdown and synthesis go on constantly with a continuing supply of α-glycerophosphate required for the latter process. When α-glycerophosphate production becomes sufficiently limited, esterification no longer keeps pace with lipolysis and net accumulation of FFA results. Conversely, when glucose utilization reaches a certain level, the rate of esterification exceeds that of lipolysis and there is a net decrease in FFA in the system. There is very little fatty acid synthesis until glucose utilization has reached a level at which production of glycerophosphate is approaching a maximum. In a descriptive sense, this may mean that in the intact organism glucose is converted to fat for storage only after the requirements of the tissue for retention of the fatty acids already present are met. This description obviously does not take into account changes in the rate of lipolysis. It is not known whether glucose metabolism itself can influence the rate of triglyceride breakdown.

Metabolism of Other Sugars. Using a manometric measure of over-all changes in gas pressure, Ball and Cooper (9) were able to demonstrate an effect of insulin on the utilization of mannose and fructose as well as of glucose by adipose tissue. The pattern of metabolism of uniformly labeled C^{14}-mannose was observed by Wood *et al.* (101) to be similar to that of glucose. In general, the amount of mannose used for synthesis of CO_2, fatty acids, glyceride glycerol, and glycogen was found to be 60% to 90% of that of glucose. It might be concluded that this difference is conditioned either by a difference in the rate of entry into the cell, or by the rate of phosphorylation prior to further metabolism. In the view of Wood and co-workers (101), the available evidence favors the latter possibility.

Glucose oxidation in adipose tissue is almost completely blocked by 2-deoxy-glucose. A glucose analogue, 6-deoxy-6-fluoroglucose, that presumably cannot be phosphorylated by hexokinase, also inhibits the oxidation of glucose but the inhibition is not complete. In adipose tissue it reaches a maximum at about 70% inhibition, when further increases in inhibitor concentration have little effect. These findings, which are qualitatively similar to those obtained in other tissues with 6-deoxy-6-fluoroglucose, have been interpreted to mean that there are two pathways for glucose uptake by cells, only one of which is inhibited by this analogue (102, 103).

ENZYMATIC PATHWAYS OF CARBOHYDRATE METABOLISM

Glycogen Synthesis and Degradation, Including the Phosphorylase System. A summary of the enzymatic reactions involved in glycogen synthesis and degradation is found in Figure 2. Though the phosphorylation of glucose has not been studied in homogenates of adipose tissue, hexokinase (*a*) is undoubtedly present. As indicated by the blocked arrow, there is apparently no glucose-6-phosphatase (*b*) activity in adipose tissue (18, 104). Phosphoglucomutase (*c*) has been assayed (104, 105) and, as in most other tissues, it appears to

exceed in activity the other enzymes involved in glycogen synthesis and degradation (105). In all tissues where it has been investigated there appear to be separate pathways for the formation and the breakdown of glycogen. Leloir and Cardini (106) have described an enzyme, UDPG glycogen transglucosylase (*e*), that catalyzes the synthesis of glycogen from uridine diphosphate glucose (UDPG). This enzyme, although it is very likely present in adipose tissue, has not been directly demonstrated. UDPG pyrophosphorylase (*d*) has been assayed in adipose tissue (105).

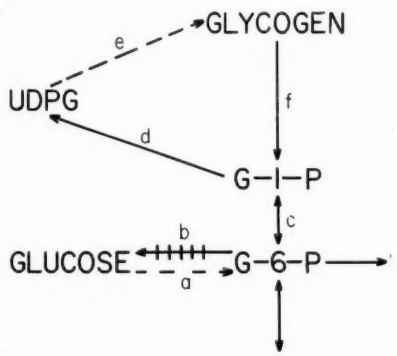

FIG. 2. Pathways of glycogen metabolism.

The reaction catalyzed by phosphorylase (*f*), an enzyme that has been extensively studied in several tissues, was long considered to be involved in both the synthesis and degradation of glycogen, although several indisputable physiological facts were difficult to integrate into such a picture. Most of these latter observations can be readily explained within the framework of the present view that glycogen synthesis proceeds via reactions (*d*) and (*e*), whereas phosphorylase (*f*) is involved only with the degradation of glycogen. The importance of the role of phosphorylase in adipose tissue is difficult to assess. Tuerkischer and Wertheimer (107) found no detectable glycogen in tissues from normally fed rats. Transient accumulation of glycogen in the adipose tissue was demonstrable shortly after consumption of a carbohydrate-rich feeding, particularly if this was preceded by a period of fasting. In a recent study the glycogen content of fat was reported as 0.22 mg/g in tissue from fed rats and 0.11 mg/g in tissues from fasted rats (105). In the absence of substrate it is difficult to understand what effect alterations in phosphorylase activity per se may have on tissue metabolism.

Figure 3 is a schematic representation of the reactions involved in the regulation of phosphorylase activity. Adipose tissue phosphorylase has not been isolated and characterized. Thus, the intimate mechanism of its inactivation and reactivation is not known. The enzyme is rapidly inactivated when intact or homogenized adipose tissue is incubated *in vitro*. This inactivation is inhibited by fluoride (18). In this and in other of its characteristics the adipose tissue system is similar to that in liver described by Sutherland and co-workers (108, 109) and differs from the muscle system.

The enzyme that inactivates phosphorylase in liver is a phosphatase and inactive phosphorylase is dephospho-phosphorylase (108). The nature of the inactivating enzyme in adipose tissue has not been demonstrated. Likewise, the mechanism of reactivation of phosphorylase in adipose tissue is not known. In the liver system reactivation consists in phosphorylation of the inactive enzyme by ATP catalyzed by a phosphokinase (109). The reaction is accelerated by cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) (110). The formation of 3′,5′-AMP in a particulate fraction of adipose tissue homogenate has been demonstrated (18, 111). Accumulation of 3′,5′-AMP in this system is enhanced by epinephrine as it is in liver, skeletal muscle, cardiac muscle, and brain (111). The mechanism of the effect of epinephrine (and of other active catecholamines) is not known. Sutherland and co-workers (111) have also demonstrated that the diesterase that inactivates 3′,5′-AMP is present in adipose tissue.

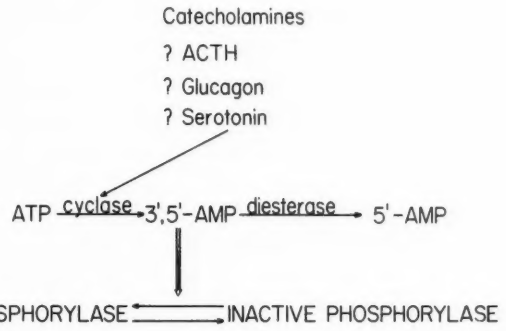

FIG. 3. Reactions involving phosphorylase and cyclic 3′,5′-AMP.

It is presumed that the stimulation of phosphorylase activity in adipose tissue induced with epinephrine or one of several other hormones is mediated by 3′,5′-AMP. Addition of 3′,5′-AMP to the medium in which tissues are incubated causes, however, none of the changes produced by epinephrine under similar conditions, including the stimulation of phosphorylase activity (18, 31). This does not, of course, exclude the possibility that the nucleotide formed *within* the tissue

plays a role in the effects of epinephrine and certain other hormones.

It should not be concluded that the action of 3′,5′-AMP and/or its formation or degradation is related necessarily to phosphorylase only. The information is summarized here since this is the context in which the system was discovered and its workings elucidated. It is quite probable that 3′,5′-AMP and/or the processes related to its formation and removal will be found to have effects on cellular function in addition to its well-documented effects in the phosphorylase system. It has been reported, for example, that this nucleotide enhances the activity of phosphofructokinase in extracts of the liver fluke, Fasciola hepatica (112).

Glucose Dissimilation and Oxidation, Including α-Glycerophosphate Synthesis and Breakdown. In Figure 4 are summarized some of the enzymatic reactions discussed below.

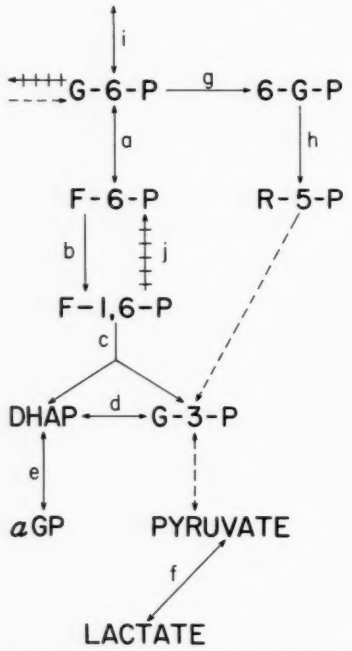

FIG. 4. Some pathways of glucose metabolism in adipose tissue.

Studies of the metabolism of differentially labeled glucose in intact adipose tissues have indicated that the oxidative pathway plays an important part in glucose metabolism. Glucose-6-phosphate dehydrogenase (*g*) activity expressed per milligram tissue nitrogen is several times greater in adipose tissue than it is in liver (104). The ratios of its activity to that of 6-phosphogluconate dehydrogenase (*h*), phosphohexoseisomerase (*b*), or phosphoglucomutase (*i*) are also many times the ratios observed for the same activities in liver. Thus, in absolute amount and, perhaps more important, relative to the activity of the other enzymes associated with glucose-6-phosphate utilization, the enzyme introducing glucose-6-phosphate to the oxidative pathway is very active.

In the intact tissue the rate of triglyceride synthesis might be regulated by changes in the activity of one of the enzymes involved, by the availability of ATP or one of the other required cofactors, or by the availability of substrates. Considering the last possibility, there is probably a constant supply of fatty acids derived from lipolysis. The availability of α-glycerophosphate may, however, be more variable and, as has been suggested by several workers, may in certain circumstances be an important factor in determining the rate of glyceride synthesis. In adipose tissue, glycerophosphate cannot be formed by the phosphorylation of glycerol and thus must be formed from dihydroxyacetone phosphate, probably derived chiefly from glycolysis. It has been shown that α-glycerophosphate can be synthesized from glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate, or dihydroxyacetone phosphate by adipose tissue homogenates suitably fortified with ATP, $MgCl_2$, and DPNH. Thus, it is likely that phosphohexoseisomerase (*a*), phosphofructokinase (*b*), aldolase (*c*), triosephosphate isomerase (*d*), and α-glycerophosphate dehydrogenase (*e*) are all present in adipose tissue (78). These reactions can be observed in the supernatant solution prepared by centrifugation of homogenates at 105,000 × *g* for 60 minutes.

Lactic dehydrogenase and α-glycerophosphate dehydrogenase activity are present in roughly equivalent amounts in rat adipose tissue (113). This is true also in liver, whereas in muscle lactic dehydrogenase activity is considerably greater than that of α-glycerophosphate dehydrogenase (113). It is not known whether the amounts or relative amounts of these two enzymes play any part in determining the quantity of lactate versus α-glycerophosphate formed in tissues.

Adipose tissue homogenates hydrolyze α-glycerophosphate. There is a large fraction of activity with a pH optimum of about 5.5 and a lesser amount of activity in the alkaline range with an optimum pH about 9.5. Acid phosphatase activity is present chiefly in microsomes and in the soluble fraction. There are differences in the behavior of the acid phosphatase in these two fractions, e.g., the microsomal enzyme, in contrast to the soluble enzymes, hydrolyzes α-glycerophosphate more rapidly than it does β-glycerophosphate and is inhibited 40% by an amount of potassium

linoleate that has no effect on the soluble enzyme (78). It is not known whether in the intact cell any glycerol is formed directly from α-glycerophosphate by the action of a phosphatase or whether it is all derived from glycerides via hydrolysis.

There is good evidence that the other reactions of the oxidative pathway and the reactions between glyceraldehyde-3-phosphate and pyruvate are operative in adipose tissue but they have not been demonstrated or assayed directly. Eichel (114) has reported on the activity of several respiratory enzymes assayed in unfractionated homogenates of adipose tissue, among them cytochrome *c* oxidase, succinic oxidase, succinic dehydrogenase, lactic oxidase, lactic dehydrogenase, and DPNH cytochrome *c* reductase. He has observed also malic and isocitric dehydrogenase activities but did not find evidence of choline, glucose, glutamic or β-hydroxybutyric dehydrogenases of D-amino acid oxidase.

The possibility of an active transaldolase in adipose tissue has been suggested by Cahill *et al.* (98) as an explanation for the finding that more carbon-1 of glucose is recovered in glycerol than in fatty acids. The presence of this reaction would tend to favor the utilization of carbons 4, 5, and 6 of glucose in fatty acid synthesis. Glyceraldehyde has been shown to serve as an acceptor in transaldolase reactions (115):

Fructose-6-phosphate + D-glyceraldehyde ⇆
glyceraldehyde-3-phosphate + D-fructose

Evidence for the presence of transaldolase activity in adipose tissue has been obtained in experiments demonstrating the formation of α-glycerophosphate from glucose-6-phosphate and glyceraldehyde in the presence of DPNH but with no ATP added (78).

The apparent absence of fructose-1,6-diphosphatase has been reported (104) and would indicate that glycolysis is irreversible. This might explain the observations of Rose and Shapiro (39), who found no glycogen synthesis from acetate-1-C^{14} or from pyruvate-2-C^{14} under conditions in which uniformly labeled C^{14}-glucose was actively incorporated into glycogen.

EFFECTS OF HORMONES ADDED IN VITRO ON ADIPOSE TISSUE METABOLISM

The sensitivity of adipose tissue to a wide variety of hormones has been demonstrated in *in vitro* studies, many of which have been summarized recently by Engel and White (116). Some of these hormones, e.g., insulin and the catecholamines, undoubtedly are of basic importance in the regulation of the metabolism of this tissue in the intact animal. The physiological role of certain others is more difficult to evaluate at this time. There is no evidence, for example, that makes it possible to relate the effects of serotonin or of growth hormone as they are observed *in vitro* to the regulation of adipose tissue metabolism in the intact animal.

INSULIN

It was first demonstrated by Krahl (8) that the uptake of glucose by adipose tissue incubated *in vitro* is stimulated by the addition of insulin *in vitro*. In studies designed to provide information concerning the effects of insulin on the pathways of glucose metabolism in adipose tissue, Winegrad and Renold (47) compared the metabolic fate of C^{14} from glucose specifically labeled in carbon-1 or carbon-6. They observed, as reported previously by Milstein (117), that insulin stimulates the oxidation of carbon-1 much more than it does the oxidation of carbon-6. Fatty acid synthesis from both carbon-1 and carbon-6 is markedly increased by insulin. These workers drew attention to the striking association of large effects on fatty acid synthesis with large increases in the oxidation of carbon-1 of glucose. The correlation between the rate of glucose oxidation via the phosphogluconate pathway and the rate of lipogenesis has been frequently noted. In a discussion of lipogenesis in adipose tissue, Winegrad *et al.* (35) considered in detail the effects of insulin on fatty acid synthesis. In summary, the stimulation by insulin of fatty acid synthesis from numerous C^{14}-labeled substrates in the presence of glucose has been amply confirmed in a number of laboratories (21, 23, 48, 118). This effect, as do most other effects of insulin on adipose tissue metabolism (with exceptions as noted below), requires the presence of glucose (31, 43) and can be explained in terms of the demonstrated action of insulin on glucose uptake and metabolism.

The effect of insulin on over-all changes in gas pressure (the resultant of oxygen uptake and carbon dioxide production) during incubation of adipose tissue *in vitro* has been investigated by Ball and co-workers (9, 119 to 122). With or without glucose in the medium there is a slow decline in pressure, indicating that oxygen uptake is greater than carbon dioxide production. Within 10 minutes of the addition of insulin there is an increase in gas pressure, which continues to rise linearly with time for periods up to 3 hours. Since oxygen uptake is little changed by insulin in these experiments, it is presumed that the positive pressure response is due to the evolution of carbon dioxide, a reflection of increased lipogenesis. This effect of insulin also is demonstrable only when glucose, fructose, or mannose is present in the medium.

Insulin increases the incorporation of C^{14} from glucose

into glyceride glycerol. This effect is relatively less than that on fatty acid synthesis from glucose and is participated in to an equal extent by carbons 1 and 6 (21, 23, 48, 99). It has been presumed that the increased incorporation of C^{14} from glucose into glyceride glycerol reflects an increased rate of triglyceride synthesis. In this regard it has been shown that insulin (in the presence of glucose) decreases the rate of release of FFA (11, 18, 30, 31, 116) and stimulates the incorporation of C^{14}-labeled fatty acids into tissue glycerides (19), changes which are most simply explained as the results of an increased rate of triglyceride synthesis. It has been suggested that glucose, in its role as the major precursor of α-glycerophosphate (and perhaps by providing a supply of energy for the esterification processes), is of basic importance in the regulation of triglyceride synthesis (see above).

In general, the pattern of glucose metabolism is essentially the same whether glucose uptake is stimulated by insulin or by increasing the concentration of glucose in the medium (48). Fatty acid synthesis from both carbon-1 and carbon-6 is greatly increased. The incorporation of both of these carbons into glycerol is enhanced but to a lesser degree. Oxidation of carbon-1 to CO_2 is markedly stimulated and oxidation of carbon-6 only slightly stimulated. These findings are compatible with the view that the action of insulin is exerted before the divergence of the metabolic pathways for glucose, i.e., at the site of entry of glucose into the cell or of glucose phosphorylation. Data from studies of the effect of insulin on incorporation of C^{14} from mannose into glycogen, carbon dioxide, fatty acids, and glyceride glycerol is also consistent with this hypothesis (101). The effects of insulin on glycogen synthesis, however, seem to be more specific and may require another explanation. When equivalent increases in glucose uptake are produced by increasing glucose concentration, or by prolactin or insulin, only in the last instance is there a significant increase in the incorporation of C^{14}-glucose into glycogen. Stimulation of glucose uptake and glycogen synthesis (nonradioactive) by tolbutamide *in vitro* has been reported (123).

Stimulation by insulin of lactic acid formation from glucose can be observed under certain conditions (122). In the presence of glucose, incorporation of C^{14}-glycine into adipose tissue protein is also enhanced by insulin (124).

Adipose tissue *in vitro* is remarkably sensitive to insulin. As little as 10 micro units/ml stimulates the oxidation of glucose-1-C^{14} (125). With an assay method based on this effect, it has been found that adipose tissue is influenced by insulinlike activity present in serum to which the rat diaphragm *in vitro* is insensitive (126). After incubating serum with extracts of adipose tissue, insulinlike activity in the diaphragm assay can be demonstrated. A fraction of adipose tissue homogenate containing the "insulin-activating" component has been prepared using ethanol fractionation and paper electrophoresis. Neither the nature of the "inactive" insulin nor the mechanism of action of the adipose tissue extracts is known (127).

In a few instances effects of insulin have been demonstrated in the absence of glucose. These obviously cannot be explained as the result of enhancement of glucose uptake. Haugaard and Marsh (128) observed a relatively small but a significant stimulation of oxygen uptake by insulin when the medium contained either glucose, succinate, lactate, acetate, or pyruvate. There was no stimulation of oxygen uptake in the absence of substrate. Increased incorporation of C^{14} from pyruvate-3-C^{14} or from acetate-1-C^{14} into adipose tissue protein by insulin in the absence of glucose has been reported. Incorporation of C^{14} into lipids determined in the same experiments was not affected (129).

Barnett and Ball (130 to 133) have explored the effects of insulin on the morphology of adipose tissue cells as revealed by electron microscopy. Tissues incubated for as little as 10 minutes in medium containing insulin exhibit invaginations of the plasma membrane, collections of numerous minute vesicles in the region of the plasma membrane, and occasionally what appeared to be long channels lined with membrane and connecting with the surface membrane. This picture is not seen in tissues incubated without insulin. The authors have suggested that the morphologic changes represent pinocytosis, the result of insulin action. These changes are produced by insulin whether or not glucose is present in the medium.

The available evidence suggests, as has been stated by Winegrad and Renold, that "adipose tissue is a major anatomical site of insulin action" (36). The mechanism of action of insulin in adipose tissue is likely very similar to that in other tissues and remains to be elucidated. As in other tissues where insulin effects have been studied, an action of insulin facilitating the entry of glucose (or of certain other hexoses) into the metabolic machinery of the cell would account for many of its effects. But in adipose tissue, as in other tissues, there are certain observations that appear to be unexplained by such an hypothesis.

EPINEPHRINE AND NOREPINEPHRINE, INCLUDING THE EFFECTS OF 3′,5′-AMP AND 5′-AMP

Epinephrine has been used in *in vitro* studies more extensively than has norepinephrine. They are ap-

parently alike in actions and in effective concentrations and will be discussed together. The data are presented in some detail, and because they are descriptive also of the effects of ACTH, the section on the latter hormone is brief and refers frequently to this one. The effects of glucagon are similar to those of the catecholamines and ACTH and will be discussed within the same framework.

The effects of epinephrine in adipose tissue metabolism are several. Since the mechanism through which they are produced is unknown and since it is not clear at present in what manner some of the apparently diverse actions are related, they will be considered separately: (*a*) Effects on fatty acid release, lipolysis, and triglyceride synthesis, (*b*) effects on glucose uptake and metabolism, and (*c*) effects on phosphorylase activity and 3′,5′-AMP.

In the early studies of Gordon and Cherkes (20) and of White and Engel (16, 17) it was demonstrated that fatty acid release is stimulated by the addition of epinephrine or norepinephrine to the medium *in vitro*. The concentration of FFA in epinephrine-treated tissues rises before accumulation in the medium is evident, and at the end of the incubation period the tissue FFA concentration is higher in the epinephrine-treated tissues than it is in the controls (16 to 19). The magnitude of the epinephrine effect is not altered by incubation in nitrogen[9] (21) or by the additon of 5×10^{-3} M iodoacetate (21). It has been shown that addition to the medium of phentolamine, dibenamine, dibenzylene, or dihydroergocornine[9] (31, 134) inhibits the effects of the catecholamines on FFA release.

Since triglyceride breakdown and resynthesis goes on constantly in adipose tissue, producing and removing FFA, alteration in the rate of either of the processes should lead to a net change in the amount of FFA in the system. The decreased rate of fatty acid release induced by the presence of glucose probably results in large part from an increase in the rate of triglyceride synthesis. The effects of epinephrine on the rate of triglyceride synthesis have been difficult to assess and will be considered after discussing the evidence for what is probably the chief factor in the increased fatty acid release, an increased rate of lipolysis.

When adipose tissue is incubated *in vitro*, glycerol (21, 32) as well as FFA is liberated into the medium. These are the products of hydrolysis of triglycerides. As discussed above, the fatty acids can be re-esterified, the rate of this process depending probably to a large extent on the supply of α-glycerophosphate. Glycerol is not metabolized to any significant extent[9] (27, 46). The amount of glycerol accumulated during an incubation is therefore an indication of the amount of lipolysis, whereas the amount of FFA accumulated represents the difference between the amount formed and the amount removed. When release of FFA is increased by the addition of epinephrine, the amount of glycerol released is also increased, strongly suggesting that the rate of triglyceride breakdown is increased (21, 32). The only other apparent source of glycerol in adipose tissue is α-glycerophosphate. The presence of α-glycerophosphatase activity has been demonstrated (78) but it is not known whether this reaction is operative in intact adipose tissue. Furthermore, the concentration of α-glycerophosphate in adipose tissue is low (0.1 to 0.2 μmole/g tissue wet weight)[8] and the effect of epinephrine on glycerol release has been observed in the absence of glucose in the medium, i.e., when it is presumed there is little synthesis of α-glycerophosphate. Lynn *et al.* (21) have found that the glycerol released from tissues incubated with C^{14}-glucose and epinephrine contained no radioactivity, strong evidence that it came from triglyceride, not α-glycerophosphate.

Positive evidence in support of the postulated effect of epinephrine on lipolysis is provided by the work of Rizack (95) and of Hollenberg *et al.* (96), who have observed increased lipase activity on adipose tissues incubated with epinephrine (and with ACTH). In summary, it has been found that when epididymal fat pads are incubated with one of these hormones, then homogenized, and the lipase assayed in a system containing buffer (pH about 7), albumin, and an artificial coconut oil emulsion, the activity is higher than it is in control tissues incubated without hormone. Rizack has reported also that the lipolytic system in a cell-free extract of adipose tissue can be activated by incubating the extract with epinephrine and either ATP or tissue sediment. The activity of this lipase in homogenates is inhibited by sodium fluoride, as is the effect of epinephrine (or ACTH) on FFA release in intact tissues (96). A role for this lipase in the action of epinephrine is strongly suggested. The magnitude of the changes in activity induced by the hormone seems hardly great enough to account for the large increases in the rate of fatty acid release in intact tissues. However, this may mean only that the conditions thus far employed are not optimal for demonstration of changes in the activity of the specific lipase affected by epinephrine.

It has been shown that the incorporation of palmitic acid-1-C^{14} into glycerides in the epididymal fat pad is markedly decreased in the presence of epinephrine (29). The tissues incubated with epinephrine contained more FFA than did the controls in these studies, but they also contained more C^{14}-palmitate and the specific activity of the total tissue FFA was actually higher in the epi-

[9] D. Steinberg and M. Vaughan, unpublished observations.

nephrine-treated tissues. The specific activity of the medium FFA was, on the other hand, considerably lower in the flasks containing epinephrine than it was in the control flasks. Steinberg *et al.* (29) have discussed the problems in interpretation presented by these experiments. For reasons discussed in detail above, it is impossible to calculate in experiments such as these the rate of fatty acid esterification, nor is it reasonable to assume that when changes in the rate of incorporation of labeled fatty acid are induced, they are necessarily proportional to alterations in the rate of fatty acid esterification. The specific activity of the precursor pool of fatty acids for triglyceride synthesis is not known at any time during the course of the experiment. It has not been isolated and determined nor can it be predicted.

It has been shown that the incorporation of glycerol-1,3-C^{14} and of pyruvate-1-C^{14} into triglycerides by adipose tissue *in vitro* is decreased by epinephrine[9] (29, 46). The interpretation of these data also is complicated by the question of precursor specific activity. Leboeuf *et al.* (32) have postulated that triglyceride synthesis is increased by epinephrine on the basis of the observation that epinephrine increased the incorporation of C^{14} from glucose into glyceride glycerol. The specific activity of α-glycerophosphate from which glyceride glycerol is formed is not known in these studies and the possibility cannot be excluded that changes in the amount of C^{14} found in glyceride glycerol are the result of changes in precursor specific activity.

Prior to considering the effects of the catecholamines on glucose metabolism it may be pertinent to point out that the effects of these hormones on fatty acid release (31), on phosphorylase activity (18), on incorporation of glycine-1-C^{14} into protein (124), on lactic acid production (121), and the characteristic effects on net gas exchange (121) are evident whether or not glucose is present in the medium.

The uptake of glucose by adipose tissue is increased in the presence of epinephrine or norepinephrine (21, 31, 32). It is possible, however, with low concentrations of these hormones (0.1 μg/ml) to produce large effects on fatty acid release with no measurable change in glucose uptake (31).

Amounts of epinephrine that cause little or no increase in glucose uptake do have marked effects on the pattern of glucose metabolism as observed in studies with C^{14}-labeled glucose (31). Carbon dioxide production from glucose-6-C^{14} is stimulated to a greater degree by epinephrine than is oxidation of glucose-1-C^{14} (21, 23). In tissues from fed rats incubated with uniformly labeled C^{14}-glucose, the most striking effect of epinephrine is on the incorporation of C^{14} into glyceride glycerol, which is markedly increased (23). In tissues from fasted rats in which fatty acid synthesis from glucose is already low, the most consistent effect of epinephrine is to decrease the incorporation of glucose carbon into fatty acids, although an increased incorporation into glyceride glycerol is also evident (31). In tissues from fed rats incubated with insulin, it has been shown that the addition of epinephrine decreases synthesis of fatty acid from labeled glucose (23). Incorporation of acetate-1-C^{14} from medium containing glucose into total lipid of adipose tissue has been found to be inhibited by epinephrine (118).

It has been suggested that the effects of epinephrine (and ACTH and growth hormone) on C^{14}-glucose metabolism might be the result of the elevated concentration of FFA in the tissue produced by these hormones (23, 135). By increasing the concentration of fatty acid in the incubation medium it is possible to produce an increase in the incorporation of glucose carbon into glyceride glycerol, i.e., an effect qualitatively like those of the hormones mentioned (23, 135).

It has been found that in the presence of epinephrine, fatty acid release can be significantly inhibited by dihydroergocornine with no effect on the incorporation of glucose carbon into glycerides (31). In these experiments, however, the concentrations of FFA in the ergocornine-treated tissues were still above the usual control levels, perhaps sufficiently elevated to affect glucose metabolism. Certainly the relationship (not necessarily causal) between FFA release and the pattern of glucose metabolism is indicated by the observations that (*a*) glucose does not abolish FFA release in the presence of epinephrine as it does in its absence (31), and (*b*) in C^{14}-glucose experiments, when both epinephrine and insulin are present, the "epinephrine pattern" of metabolism of C^{14}-glucose prevails (23).

Epinephrine and norepinephrine both increase the amount of active phosphorylase in adipose tissue (18). It is not clear what effect this has on adipose tissue metabolism since the amounts of glycogen present in the tissue under most circumstances are minimal and no other substrate for phosphorylase is known. The increase in phosphorylase activity is mediated by the action of 3′,5′-AMP, the production of which is accelerated by epinephrine (see above). It is quite possible that 3′,5′-AMP has effects in addition to its action on phosphorylase. Either these or effects resulting from the formation of 3′,5′-AMP from ATP and its subsequent hydrolysis may account for some of the metabolic changes produced by epinephrine. The production of 3′,5′-AMP and of active phosphorylase is clearly not related directly to stimulation of FFA release and effects on pathways of glucose metabolism, since when

serotonin produces the former effects in adipose tissue, there is no change in FFA release or in the pattern of C^{14}-glucose metabolism (31). Serotonin does, however, increase glucose uptake (31). Addition of 3′,5′-AMP to the medium does not increase phosphorylase activity in adipose tissue nor does it reproduce any of the other effects of epinephrine (18). Both 3′,5′-AMP and 5′-AMP (at equal concentrations) decrease fatty acid release and stimulate glucose uptake slightly. However, they enhance the incorporation of C^{14} from uniformly labeled C^{14}-glucose into both moieties of the triglyceride, i.e., fatty acids and glycerol, to an equal extent (18, 31).

When 3′,5′-AMP is inactivated in tissues, the product is 5′-AMP. This reaction is inhibited by caffeine. It has not been possible, however, to use caffeine inhibition to determine whether the effects observed with 3′,5′-AMP are all due to its conversion to 5′-AMP by the tissue since caffeine itself affects the adipose tissue. It inhibits glucose uptake and causes a slight stimulation of FFA release (31). Thus, the data available at present are inconclusive with respect to the role played by 3′,5′-AMP (or its synthesis or degradation) in production of most of the effects of epinephrine in adipose tissue.

Epinephrine enhances the accumulation of 3′,5′-AMP, which in turn increases the amount of active phosphorylase and may be involved in the stimulation of glucose uptake. It is not known whether the latter effect could result from an increase in phosphofructokinase activity, which has been produced with 3′,5′-AMP in extracts of Fasciola hepatica (136) but has not been demonstrated in adipose tissue. Rizack (95) has suggested that 3′,5′-AMP is not the mediator of the epinephrine-induced increase in lipase activity which most likely is the cause of increased FFA release. It is possible that the accumulation of FFA within the tissue influences cellular metabolism and that this is reflected in changes in the pattern of C^{14}-glucose metabolism.

ADRENOCORTICOTROPIC HORMONE (ACTH)

The effects of ACTH on the metabolism of adipose tissue *in vitro* are qualitatively and quantitatively like those of the catecholamines. One difference is that the effect of ACTH on the release of FFA *in vitro* cannot be demonstrated in medium containing no calcium, whereas the effect of epinephrine does not require the presence of calcium (137). It is of interest that calcium is also required for the effect of ACTH steroid production by adrenal slices (138).

When ACTH is added to adipose tissue *in vitro*, the release of FFA and the concentration of FFA in the tissue are increased (16, 17, 18, 137). Lipase activity is increased (96) and incorporation of palmitic acid-1-C^{14} into glycerides is decreased (29). Glucose uptake is stimulated (31, 48, 135). The effects of ACTH on the incorporation of glucose carbon into glyceride glycerol and into fatty acids mimic those of epinephrine (31, 48, 135). Incorporation of acetate-1-C^{14} into total lipids is also depressed by ACTH in medium containing glucose (118). Phosphorylase activity is increased presumably through the mediation of 3′,5′-AMP (18).

Phenotolamine *in vitro* blocks the effect of ACTH (just as it does the effects of catecholamines) on the release of FFA (134). When the effect on FFA release has been partially inhibited, there is no change in the ACTH induced pattern of C^{14}-glucose metabolism (31).

The relationship of peptide structure to actions of ACTH in different tissues is of interest. Engel and White (116) have summarized data suggesting that the N-terminal serine of the ACTH molecule may be required for its adrenal effects but not for the effects on adipose tissue. α-Melanophore stimulating hormone, a peptide consisting of 18 amino acids identical in sequence with the N-terminal portion of the ACTH molecule, causes release of FFA from adipose tissue but is not as potent in this regard as is ACTH. The activities of a group of related peptides have also been examined.

It is rather surprising to find that molecules as different in structure as a catecholamine and ACTH, a polypeptide, produce apparently identical effects. It is difficult to visualize a single enzyme or "receptor" that would be capable of interacting with two molecules of such dissimilar configuration. It is almost necessary to conclude that they do not act at the same site. This conclusion is favored by the recent work of Paoletti and Smith,[10] who have found that the effects of ACTH on FFA release are not obtained in adipose tissue that is depleted of catecholamines (chiefly norepinephrine). Their data strongly suggest that norepinephrine stored in the tissue is in some way "released" by the action of ACTH and produces the effects attributed to the latter hormone.

GLUCAGON

The effects of glucagon resemble in many ways those of ACTH and the catecholamines but differ in some particulars. The rate of release and the tissue concentration of FFA are increased by glucagon whether or not glucose is present in the medium (18, 22, 31, 139). The maximal effect of glucagon is, however, much

[10] R. Paoletti and R. L. Smith, personal communication.

smaller than those obtained with epinephrine or ACTH. Glycerol release is also increased (22), suggesting an increased rate of lipolysis.

Glucagon stimulates glucose uptake (22, 31, 140, 141). Its effect is somewhat greater than those of epinephrine and ACTH. As with the latter hormones, it is possible to demonstrate effects on the release of FFA with concentrations of glucagon that do not cause a measurable increase in glucose uptake (31). It is of interest to know whether any or all of the effect of glucagon on glucose uptake might be due to the presence of insulin in the glucagon preparations. The effect of glucagon on glucose uptake is not altered by treatment of the hormone with cysteine, which inactivates insulin (31, 140), although Lee and co-workers (140) have reported that the insulinlike effect of glucagon on the oxidation carbon-1 of glucose is diminished by cysteine treatment. Hagen (22) has found that the addition of insulin to an amount of glucagon that is maximally effective in stimulating of glucose uptake causes a further increase. She has also observed that the stimulation of oxygen uptake caused by glucagon (in the presence of glucose but not in its absence) is enhanced by the addition of insulin, which alone has little effect. Insulin also enhances the stimulatory effect of epinephrine on oxygen uptake. All of these findings support the view that the effects on glucose metabolism are due to glucagon itself, not to traces of insulin.

Glucagon increases the incorporation of C^{14} from uniformly labeled C^{14}-glucose into glyceride glycerol and causes a more variable decrease in incorporation into fatty acids in tissues from fed rats (141). Inhibition of lipid synthesis from acetate-1-C^{14} has been observed (118). With tissues from fasted rats, glucagon has little effect on fatty acid synthesis from uniformly labeled C^{14}-glucose but greatly enhances glyceride glycerol formation (31). These findings differ at least quantitatively from the effects of epinephrine and of ACTH observed under similar conditions but are clearly not like those of insulin (31). In terms of the hypothesis of Cahill *et al.* (23), concerning the part played by tissue fatty acids in determining the pattern of glucose metabolism, it is possible that differences in the effects of glucagon and of epinephrine, e.g., on the fate of C^{14}-labeled glucose, are due to differences in extent of FFA accumulation induced by the two hormones (18).

Like epinephrine, norepinephrine, ACTH, and serotonin, glucagon increases phosphorylase activity in adipose tissue (18, 22). As mentioned in the discussion of the effects of ACTH, one wonders that so many different molecules produce what appear to be the same effects. It seems probable that this is not due to a lack of specificity in some key reaction. More likely, it will be found, as is suggested by the evidence available concerning ACTH,[10] that the "receptor" or primary site of action is different for each of these hormones.

SEROTONIN

The effects of serotonin on adipose tissue have not been investigated in detail. It increases phosphorylase activity and stimulates glucose uptake when added to the medium in which intact fat pads are incubated (18, 31). Similar effects of serotonin on the liver fluke, F. hepatica, have been reported by Mansour and Lago (142), who have established that serotonin in the fluke enhances the accumulation of 3′,5′-AMP, which in turn mediates the effects on phosphorylase activity (112). It is probable that the effects on adipose tissue phosphorylase are similarly produced. The mechanism of the effect on glucose uptake is less clear. It has been shown that 3,5′-AMP increases phosphofructokinase activity in extracts of F. hepatica (136) but it is not established that such an action would lead to an increase in glucose uptake. Nor is it known whether fructokinase in adipose tissue is similarly affected by 3′,5′-AMP.

Serotonin has proved most interesting because of its lack of effect on release of FFA from adipose tissue (18). Epinephrine, norepinephrine, ACTH, and glucagon, all of which enhance phosphorylase activity in this tissue apparently through the mediation 3′,5′-AMP, also stimulate the release of FFA. The observations with serotonin, therefore, indicate that either the effects of the several hormones on the release of FFA are unrelated to their influence on the 3′,5′-AMP-phosphorylase system or that serotonin, in addition to this action shared with the other hormones, in some manner interferes with fatty acid release. No evidence for the latter alternative has been obtained in an investigation of the effects of serotonin on the release of FFA from adipose tissue in the presence of epinephrine.[6]

SOME PITUITARY HORMONES THAT HAVE EFFECTS ON ADIPOSE TISSUE

Information concerning several pituitary hormones that have effects on adipose tissue metabolism is briefly summarized here. The effects of these hormones *in vitro* have not been extensively studied and in many cases the preparations employed are impure or possibly impure. Thus, it is not possible to draw any conclusions about the mechanism of action or the part played by these hormones in adipose tissue metabolism.

Growth Hormone. The release of FFA from adipose tissue *in vitro* is stimulated by growth hormone (17,

135). High concentrations of the hormone are required to obtain this effect, although the injection of growth hormone in the intact animal raises serum FFA concentration more dramatically than does ACTH.

It has been found that when glucose is present in the medium, oxygen uptake is increased by the addition of growth hormone (122). Glucose uptake is stimulated but there is no increase in lactic acid production (122). The effects of growth hormone preparations on the pathways of glucose metabolism are complex and difficult to interpret. Leboeuf and Cahill (135) have concluded that the action of growth hormone on glucose metabolism is like that of ACTH and have suggested that it is the result of the accumulation of FFA in tissues incubated with the hormone. Winegrad and coworkers (35, 99) have observed that growth hormone preferentially increases the oxidation of carbon-6 of glucose with little effect on the oxidation of carbon-1. On the basis of their data they have suggested that growth hormone stimulated the activity of the glucuronic acid pathway. It has been found also that fatty acid synthesis from acetate-1-C^{14} or from pyruvate-2-C^{14} is decreased by growth hormone whether or not glucose is present in the medium (35, 118). This and several other effects of growth hormone on adipose tissue metabolism are apparently similar to those of epinephrine and ACTH. Growth hormone does not, however, increase lipase activity as do the latter two hormones (96) and differs in other characteristics as well.

Fat-Mobilizing Factor from Human Urine. Chalmers *et al.* (143, 144, 145) have isolated from the urine of fasting humans a fraction which, when injected into mice, causes an increase in liver fat, blood lipids, and blood ketones. The active principle has been purified by these workers to the extent that as little as 1 μg of the purified material per milliliter of incubation medium stimulates the release of FFA from adipose tissue *in vitro*. This fat-mobilizing factor is apparently a relatively small peptide. Its effects, both *in vivo* and *in vitro*, are in many ways similar to those of ACTH but it differs in chemical characteristics from ACTH. It is much more active than growth hormones in stimulating fatty acid release *in vitro* and its effects *in vivo* are not like those of growth hormone.

Fasting does not induce the urinary excretion of the active factor in patients with pituitary deficiency or in hypophysectomized goats. The administration of human growth hormone to two patients with pituitary deficiency did not lead to the appearance of activity in the urine. Fat-mobilizing factor activity has been found in the urine of fasting humans, goats, sheep, horses, and dogs. Weil and Stetten (146) had reported earlier the presence of what is probably a similar material in the urine of fasting rabbits. It is not present in the urine of normal humans consuming a regular diet but has been found in the urine of persons consuming a diet restricted in calories and very low in carbohydrate. The administration of ACTH to nonfasting subjects does not lead to the appearance of fat-mobilizing activity in the urine.

Much remains to be learned about this factor and its physiological role in the regulation of adipose tissue metabolism. The only information in the literature at present concerns its effects on the release of FFA.

Pituitary Fraction H. Rudman and co-workers have described the presence in extracts of hog pituitary gland of a component, Fraction H, which differs from previously described pituitary hormones (147, 148, 149). When injected into rabbits, this material causes a marked increase of serum FFA within 2 hours and an increase in total blood lipids 4 to 10 hours after injection (149, 150). Less than 1 mg of the purified Fraction H is required to produce these effects. Stimulation *in vitro* of the release of FFA from rabbit adipose tissue has been demonstrated with this material. (The effectiveness of Factor H from hog pituitary varies markedly with the species of the test animal.) There is no further information presently available concerning its action in adipose tissue.

A preparation of Factor H that behaves as a single component on paper electrophoresis has been obtained by Rudman and co-workers (151). The active substance is nondialyzable, insoluble in 5% trichloroacetic acid, and is destroyed by trypsin. Activity survives treatment with pepsin but is then found to be dialyzable and soluble in 5% trichloroacetic acid. Fraction H activity appears to reside in a relatively small molecule that, when isolated, is either an integral part of, or tightly bound to, a larger protein molecule (151).

Prolactin, Thyrotropin, and Follicle-Stimulating Hormone. Prolactin at concentrations up to 100 μg/ml has been found to be without effect on the release of FFA (17). At a concentration of 1 mg/ml prolactin has been shown to affect glucose metabolism in adipose tissue in a manner that much resembles that of insulin (35, 36, 99). When prolactin is present, oxidation of carbon-1 is markedly increased, with a much smaller increase in the rate of CO_2 formation from carbon-6. Fatty acid synthesis from C^{14}-glucose (or from acetate-1-C^{14} or pyruvate-2-C^{14} in the presence of glucose) is greatly enhanced (35, 36). Prolactin does not, however, stimulate glycogen synthesis from glucose to the extent that insulin does (35, 36). In addition, although glucose uptake by adipose tissue from alloxan diabetic rats is increased by prolactin, this hormone does not

alleviate the defect in fatty acid synthesis as does insulin (35).

White and Engel (17) have reported that the release of FFA from adipose tissue *in vitro* is stimulated by thyrotropin and by follicle-stimulating hormone in high concentrations. As has been pointed out by the authors, the hormone preparations that were used were impure. Thus, the significance of the effects is difficult to evaluate.

REFERENCES

1. Wertheimer, E., and B. Shapiro. *Physiol. Revs.* **28**: 451, 1948.
2. Favarger, P. *Helv. Physiol. et Pharmacol. Acta* **7**: C-41, 1949.
3. Gordon, R. S., Jr., and A. Cherkes. *J. Clin. Invest.* **35**: 206, 1956.
4. Gordon, R. S., Jr. *J. Clin. Invest.* **36**: 810, 1957.
5. Dole, V. P. *J. Clin. Invest.* **35**: 150, 1956.
6. Shapiro, B., and E. Wertheimer. *J. Biol. Chem.* **173**: 725, 1948.
7. Hausberger, F. X., and S. W. Milstein. *J. Biol. Chem.* **214**: 483, 1955.
8. Krahl, M. E. *Ann. N.Y. Acad. Sci.* **54**: 649, 1951.
9. Ball, E. G., and O. Cooper. *J. Biol. Chem.* **235**: 584, 1960.
10. McKerns, K. W., and J. R. Clynes, quoted in McKerns, K. W., and E. Kaleita. *Biochem. Biophys. Research Commun.* **2**: 344, 1960.
11. Engel, F. L., and J. E. White. *Am. J. Clin. Nutrition* **8**: 691, 1960.
12. Wertheimer, E., and E. Shafrir. In *Recent Progress in Hormone Research*, edited by G. Pincus, New York and London, Academic Press, Inc., 1960, vol. 16, p. 467.
13. Wertheimer, E., M. Hamosh and E. Shafrir. *Am. J. Clin. Nutrition* **8**: 705, 1960.
14. Goodman, D. S. *J. Am. Chem. Soc.* **80**: 3892, 1958.
15. Reshef, L., E. Shafrir and B. Shapiro. *Metabolism* **7**: 723, 1958.
16. White, J. E., and F. L. Engel. *Proc. Soc. Exptl. Biol. Med.* **99**: 375, 1958.
17. White, J. E., and F. L. Engel. *J. Clin. Invest.* **37**: 1556, 1958.
18. Vaughan, M. *J. Biol. Chem.* **235**: 3049, 1960.
19. Bally, P. R., G. F. Cahill, Jr., B. Leboeuf, and A. E. Renold. *J. Biol. Chem.* **235**: 333, 1960.
20. Gordon, R. S., Jr., and A. Cherkes. *Proc. Soc. Exptl. Biol. Med.* **97**: 150, 1958.
21. Lynn, W. S., R. M. MacLeod and R. H. Brown. *J. Biol. Chem.* **235**: 1904, 1960.
22. Hagen, J. H. *J. Biol. Chem.* **236**: 1023, 1961.
23. Cahill, G. F., Jr., B. Leboeuf and R. B. Flinn. *J. Biol. Chem.* **235**: 1246, 1960.
24. Leboeuf, B., and G. F. Cahill, Jr. *J. Biol. Chem.* **236**: 41, 1961.
25. Stern, I., and B. Shapiro. *Metabolism* **3**: 539, 1954.
26. Shapiro, B., I. Chowers and G. Rose. Second International Conference on Biochemical Problems of Lipids, Ghent, 1955.
27. Shapiro, B., I. Chowers and G. Rose. *Biochim. et Biophys. Acta* **23**: 115, 1957.
28. Shapiro, B. *Prog. in Chem. Fats Lipids* **4**: 178, 1957.
29. Steinberg, D., M. Vaughan and S. Margolis. *J. Biol. Chem.* **235**: PC 38, 1960.
30. Raben, M. S., and C. H. Hollenberg. *J. Clin. Invest.* **39**: 435, 1960.
31. Vaughan, M. *J. Biol. Chem.* In press.
32. Leboeuf, B., R. B. Flinn and G. F. Cahill, Jr. *Proc. Soc. Exptl. Biol. Med.* **102**: 527, 1959.
33. Marinetti, G. V., and E. Stotz. *Biochim. et Biophys. Acta* **21**: 168, 1956.
34. Kerpel, S., E. Shafrir and B. Shapiro. *Biochim. et Biophys. Acta* **46**: 495, 1961.
35. Winegrad, A. I., W. N. Shaw, F. D. W. Lukens, and W. C. Stadie. *Am. J. Clin. Nutrition* **8**: 651, 1960.
36. Winegrad, A. I., W. N. Shaw, F. D. W. Lukens, and W. C. Stadie. *J. Biol. Chem.* **234**: 3111, 1959.
37. Favarger, P., and J. Gerlach. *Helv. Physiol. et Pharmacol. Acta* **12**: C-15, 1954.
38. Feller, D. D. *J. Biol. Chem.* **206**: 171, 1954.
39. Rose, G., and B. Shapiro. *Biochim. et Biophys. Acta* **18**: 504, 1955.
40. Miller, J. P., and J. A. D. Cooper. *Biochim. et Biophys. Acta* **24**: 430, 1957.
41. Miller, J. P., and J. A. D. Cooper. *Biochim. et Biophys. Acta* **27**: 141, 1958.
42. Miller, J. P., and J. A. D. Cooper. *Biochim. et Biophys. Acta* **33**: 436, 1959.
43. Winegrad, A. I., and A. E. Renold. *J. Biol. Chem.* **233**: 267, 1958.
44. Feller, D. D., and E. Feist. *J. Biol. Chem.* **228**: 275, 1957.
45. Feller, D. D., and E. Feist. *J. Lipid Research* **1**: 90, 1959.
46. Cahill, G. F., Jr., B. Leboeuf and A. E. Renold. *Am. J. Clin. Nutrition* **8**: 733, 1960.
47. Winegrad, A. I., and A. E. Renold. *J. Biol. Chem.* **233**: 273, 1958.
48. Jeanrenaud, B., and A. E. Renold. *J. Biol. Chem.* **234**: 3082, 1959.
49. Cahill, G. F., Jr., B. Jeanrenaud, B. Leboeuf, and A. E. Renold. *Ann. N.Y. Acad. Sci.* **82**: 403, 1959.
50. Leboeuf, B., and G. F. Cahill, Jr. *Federation Proc.* **19**: 226, 1960.
51. Masoro, E. J., A. I. Cohen and S. S. Panagos. *Am. J. Physiol.* **179**: 451, 1954.
52. Masoro, E. J., E. Porter and J. Patkin. *Am. J. Physiol.* **198**: 1123, 1960.
53. Lieber, C. S., L. M. DeCarli and R. Schmid. *Biochem. Biophys. Research Commun.* **1**: 302, 1959.
54. Renold, A. E., A. B. Hastings, F. B. Nesbett, and J. Ashmore. *J. Biol. Chem.* **213**: 135, 1955.
55. Milstein, S. W., and L. H. Driscoll. *J. Biol. Chem.* **234**: 19, 1959.
56. Korn, E. D. *Colloques Internationaux du Centre National de la Recherche Scientifique*, Marseille, 1960.
57. Rodbell, M. *J. Biol. Chem.* **235**: 1613, 1960.
58. Wakil, S. J. *J. Lipid Research* **2**: 1, 1961.
59. Ganguly, J. *Biochim. et Biophys. Acta* **40**: 110, 1960.
60. Martin, D. B., M. G. Horning and P. R. Vagelos. *J. Biol. Chem.* **236**: 663, 1961.

61. Horning, M. G., D. B. Martin, A. Karmen, and P. R. Vagelos. *J. Biol. Chem.* **236**: 669, 1961.
62. Lynen, F. *J. Cellular Comp. Physiol.* **54**: *Suppl.* **1**: 33, 1959.
63. Hele, P., G. Popják and M. Lauryssens. *Biochem. J.* **65**: 348, 1957.
64. Quagliariello, G. *Compt. rend. Acc. Linnei* **16**: 553, 1932.
65. Yosii, S. *J. Biochem.* (*Tokyo*) **26**: 397, 1937.
66. Shapiro, B., and E. Wertheimer. *Biochem. J.* **37**: 102, 1943.
67. Champougny, J., and E. LeBreton. *Compt. rend. soc. biol.* **141**: 43, 45, 1947.
68. Steinberg, D., M. Vaughan, S. Margolis, and A. Karmen. *Federation Proc.* **19**: 227, 1960.
69. Steinberg, D., M. Vaughan and S. Margolis. *J. Biol. Chem.* **236**: 1631, 1961.
70. Weiss, S. B., and E. P. Kennedy. *J. Am. Chem. Soc.* **78**: 3550, 1956.
71. Smith, S. W., S. B. Weiss and E. P. Kennedy. *J. Biol. Chem.* **228**: 915, 1957.
72. Tietz, A., and B. Shapiro. *Biochim. et Biophys. Acta* **19**: 374, 1956.
73. Stein, Y., and B. Shapiro. *Biochim. et Biophys. Acta* **24**: 197, 1957.
74. Goldman, P., and P. R. Vagelos. *J. Biol. Chem.* In press.
75. Dixon, M., and E. C. Webb. *Enzymes.* London, Longmans, Green & Co., 1958.
76. Jaenicke, L., and F. Lynen. In *The Enzymes*, edited by P. D. Boyer, H. Lardy, and K. Myrbäck, New York, Academic Press, Inc., 1960, vol. 3, p. 3.
77. Wieland, O., and M. Suyter. *Biochem. Z.* **329**: 320, 1957.
78. Margolis, S., and M. Vaughan. *J. Biol. Chem.* In press.
79. Shapiro, B., M. Statter and G. Rose. *Biochim. et Biophys. Acta* **44**: 373, 1960.
80. Quagliariello, G., and G. Scoz. *Arch. Aci. Biol.* (Italy) **17**: 513, 1932.
81. Renold, A. E., and A. Marble. *J. Biol. Chem.* **185**: 367, 1950.
82. Mashburn, L., R. Brown and W. S. Lynn. *Federation Proc.* **19**: 224, 1960.
83. Lynn, W. S., Jr., and N. C. Perryman. *J. Biol. Chem.* **235**: 1912, 1960.
84. Korn, E. D., and T. W. Quigley, Jr. *Biochim. et Biophys. Acta* **18**: 143, 1955.
85. Korn, E. D., and T. W. Quigley, Jr. *J. Biol. Chem.* **226**: 833, 1957.
86. Korn, E. D. *J. Biol. Chem.* **226**: 827, 1957.
87. Cherkes, A., and R. S. Gordon, Jr. *J. Lipid Research* **1**: 97, 1959.
88. Gordon, R. S., Jr., E. Boyle, R. K. Brown, A. Cherkes, and C. B. Anfinsen. *Proc. Soc. Exptl. Biol. Med.* **84**: 168, 1953.
89. Korn, E. D. *J. Biol. Chem.* **236**: 1638, 1961.
90. Savary, P., J. Flanzy and P. Desnuelle. *Biochim. et Biophys. Acta* **24**: 414, 1957.
91. Mattson, F. H., and E. S. Lutton. *J. Biol. Chem.* **233**: 868, 1958.
92. Robinson, D. S., and J. E. French. *Pharmacol. Revs.* **12**: 241, 1960.
93. Hollenberg, C. H. *Am. J. Physiol.* **197**: 667, 1959.
94. Robinson, D. S. Fifth International Conference on Biochemical Problems of Lipids, Marseille, 1960.
95. Rizack, M. A. *J. Biol. Chem.* **236**: 657, 1961.
96. Hollenberg, C. H., M. S. Raben and E. B. Astwood. *Endocrinology* **68**: 589, 1961.
97. Robinson, D. S. *J. Lipid Research* **1**: 332, 1960.
98. Cahill, G. F., Jr., B. Leboeuf and A. E. Renold. *J. Biol. Chem.* **234**: 2540, 1959.
99. Winegrad, A. I., W. N. Shaw, F. D. W. Lukens, W. C. Stadie, and A. E. Renold. *J. Biol. Chem.* **234**: 1922, 1959.
100. Wood, F. C., Jr., B. Leboeuf and G. F. Cahill, Jr. *Diabetes* **9**: 261, 1960.
101. Wood, F. C., Jr., B. Leboeuf, A. E. Renold, and G. F. Cahill, Jr. *J. Biol. Chem.* **236**: 18, 1961.
102. Serif, G. S., C. J. Stewart, H. I. Nakada, and A. N. Wick. *Proc. Soc. Exptl. Biol. Med.* **99**: 720, 1958.
103. Wick, A. N., and G. S. Serif. *Ann. N.Y. Acad. Sci.* **82**: 374, 1959.
104. Weber, G., G. Banerjee and J. Ashmore. *Biochem. Biophys. Research Commun.* **3**: 182, 1960.
105. Villar-Palasi, C., and J. Larner. *Arch. Biochem. Biophys.* **86**: 270, 1960.
106. Leloir, L. F., and C. E. Cardini. *J. Am. Chem. Soc.* **79**: 6340, 1957.
107. Tuerkischer, E., and E. Wertheimer. *J. Physiol.* **100**: 385, 1942.
108. Wosilait, W. D., and E. W. Sutherland. *J. Biol. Chem.* **218**: 469, 1956.
109. Rall, T. W., E. W. Sutherland and W. D. Wosilait. *J. Biol. Chem.* **218**: 483, 1956.
110. Rall, T. W., and E. W. Sutherland. *J. Biol. Chem.* **232**: 1065, 1958.
111. Sutherland, E. W., and T. W. Rall. *Pharmacol. Revs.* **12**: 265, 1960.
112. Mansour, T. E., E. W. Sutherland, T. W. Rall, and E. Bueding. *J. Biol. Chem.* **235**: 466, 1960.
113. Boxer, G. E., and C. E. Shonk. *Cancer Research* **20**: 85, 1960.
114. Eichel, H. J. *Biochem. Biophys. Research Commun.* **1**: 293, 1959.
115. Bonsignore, A., S. Pontremoli, E. Grazi, and M. Mangiarotti. *Biochem. Biophys. Research Commun.* **1**: 79, 1959.
116. Engel, F. L., and J. E. White, Jr. *Am. J. Clin. Nutrition* **8**: 691, 1960.
117. Milstein, S. W. *Proc. Soc. Exptl. Biol. Med.* **92**: 632, 1956
118. Orth, R. D., W. D. Odell and R. H. Williams. *Am. J. Physiol.* **198**: 640, 1960.
119. Ball, E. G., D. B. Martin and O. Cooper. *J. Biol. Chem.* **234**: 774, 1959.
120. Hagen, J. M., E. G. Ball and O. Cooper. *J. Biol. Chem.* **234**: 781, 1959.
121. Hagen, J. H., and E. G. Ball. *J. Biol. Chem.* **235**: 1545, 1960.
122. Jungas, R. L., and E. G. Ball. *J. Biol. Chem.* **235**: 1894, 1960.
123. R-Candela, J. L., and C. López-Quijada. *Med. Exp.* **2**: 40, 1960.
124. Herrera, M. G., and A. E. Renold. *Biochim. et Biophys. Acta* **44**: 165, 1960.

125. Renold, A. E., D. B. Martin, Y. M. Dagenais, J. Steinke, R. J. Nickerson, and M. C. Sheps. *J. Clin. Invest.* **39:** 1487, 1960.
126. Martin, D. B., A. E. Renold and Y. M. Dagenais. *Lancet* **2:** 76, 1958.
127. Antoniades, H. N., and K. Gunderson *Endocrinology* **68:** 36, 1961.
128. Haugaard, N., and J. B. Marsh. *J. Biol. Chem.* **194:** 33, 1952.
129. Krahl, M. E. *Biochim. et Biophys. Acta* **35:** 556, 1959.
130. Barrnett, R. J., and E. G. Ball. *Science* **129:** 1282, 1959.
131. Ball, E. G., and R. J. Barrnett. *Diabetes* **9:** 70, 1960.
132. Barrnett, R. J., and E. G. Ball. *Am. J. Clin. Nutrition* **8:** 666, 1960.
133. Barrnett, R. J., and E. G. Ball. *J. Biophys. Biochem. Cytol.* **8:** 83, 1960.
134. Schotz, M. C., and I. H. Page. *J. Lipid Research* **1:** 466, 1960.
135. Leboeuf, B., and G. F. Cahill, Jr. *J. Biol. Chem.* **236:** 41, 1961.
136. Mansour, T. E., and J. S. Menard. *Federation Proc.* **19:** 50, 1960.
137. Lopez, E., E. J. White and F. L. Engel. *J. Biol. Chem.* **234:** 2254, 1959.
138. Péron, F. G., and S. B. Koritz. *J. Biol. Chem.* **233:** 256, 1958.
139. Steinberg, D., E. Shafrir and M. Vaughan. *Clin. Research* **7:** 250, 1959.
140. Lee, H. M., R. M. Ellis and W. W. Bromer. *Proc. Soc. Exptl. Biol. Med.* **104:** 4, 1960.
141. Froesch, E. R., P. Bally, U. Guhl, E. Ramseier, and A. Labhart. *Schweiz. med. Wochschr.* **47:** 1329, 1960.
142. Mansour, T. E., and A. D. Lago. *J. Pharmacol. Exptl. Therap.* **122:** 48A, 1958.
143. Chalmers, T. M., A. Kekwick, G. L. S. Pawan, and I. Smith. *Lancet* **1:** 866, 1958.
144. Chalmers, T. M., G. L. S. Pawan and A. Kekwick. *Lancet* **2:** 6, 1960.
145. Chalmers, T. M., G. L. S. Pawan and A. Kekwick. *Am. J. Clin. Nutrition* **8:** 728, 1960.
146. Weil, R., and DeW. Stetten, Jr. *J. Biol. Chem.* **168:** 129, 1947.
147. Rudman, D., and F. Seidman. *Proc. Soc. Exptl. Biol. Med.* **99:** 146, 1958.
148. Rudman, D., F. Seidman and M. B. Reid. *Proc. Soc. Exptl. Biol. Med.* **103:** 315, 1960.
149. di Girolamo, M., D. Rudman, M. B. Reid, and F. Seidman. *Endocrinology* **68:** 457, 1961.
150. Rudman, D., M. di Girolamo, F. E. Kendall, A. R. Wertheim, F. Seidman, M. B. Reid, and S. Bern. *Endocrinology* **67:** 784, 1960.
151. Rudman, D., M. B. Reid, F. Seidman, M. di Girolamo, A. R. Wertheim, and S. Bern. *Endocrinology* **68:** 273, 1961.

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A study of the separation of substituted cholanic acids by gas-liquid chromatography*

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SUMMARY

Suitable conditions for the gas-liquid chromatography of a number of substituted methyl cholanates are described. The effects of various functional groups on the retention times have been studied, using as liquid phase two types of silicone gum rubbers differing in their content of phenyl groups.

The methods most commonly used for the chromatographic separation of bile acids involve reversed phase partition chromatography (1, 2, 3), partition chromatography with aqueous acetic acid as stationary phase (2, 4), and paper chromatography with acidic solvents (5, 6). The last-mentioned type of solvents was originally developed by Ahrens and Craig (7) for the countercurrent distribution of bile acids. Silicic-acid chromatography of methyl esters of bile acids has been used in some cases (8) and adsorption chromatography on impregnated glass paper (9) has proved to be very valuable for separation of extremely small amounts of bile acids. By combining several of the methods mentioned, most of the known biologically occurring bile acids can be separated. In analyses of very complicated mixtures, however, the procedures become tedious and time-consuming. Recently Vanden Heuvel *et al.* (10) showed that bile acids could be separated by gas-liquid chromatography. In connection with studies of human fecal bile acids, we have therefore further investigated the gas-liquid chromatographic behavior of a number of substituted cholanic acids. This paper describes suitable conditions for the separation of the methyl esters of some hydroxy-, acetoxy- and ketocholanic acids.

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METHODS

Gas-Chrom P, 100 to 120 mesh (Applied Science Laboratories), was used to support the stationary phase. The washing and coating of the support was carried out by the technique used by Horning *et al.* (11). Forty grams of the support was washed repeatedly with concentrated hydrochloric acid, then with distilled water, until the supernatant was neutral (fine particles were removed by decanting), and finally with acetone. After drying in an oven at 80° for 1 to 2 hours, the support was siliconized for 15 minutes with a 5% solution of dimethyldichlorosilane (General Electric Company) in toluene, under intermittent water pump vacuum to remove air from the particles. After washing with methanol until neutral, the support was dried overnight at 80°.

The stationary phases described in this work were two types of silicone rubber gum, kindly made for us by Dr. A. C. Martellock, of the Silicone Products Department, General Electric Company. They were modifications of "SE-52" and consisted of the $-(Si(CH_3)_2O)-$ and the $-(Si(C_6H_5)_2O)-$ groupings. One polymer (GE No. 287-149-243) had 20 moles per cent, and the other (GE No. 287-149-244) 35 moles per cent, of the latter groupings. One-half gram of the material was dissolved in 100 ml of toluene, 20 g of the support was added, and intermittent water pump vacuum was applied. After about 15 minutes the excess of solution was filtered off on a suction funnel and the wet support dried in an oven at 80° for 1 to 2 hours. Although the exact amount of stationary phase on the support cannot be stated, this procedure was reproducible and appeared to cause the least damage to the

support. The coated support was packed into 183 cm × 6 mm columns by tapping against the floor; packing by vibration seemed to result in tailing peaks. A Barber-Coleman Model 10 apparatus with an argon ionization detector was used. Two milligrams of the bile acids was dissolved in 0.25 ml of methanol and freshly distilled diazomethane in ether was added in excess until the solution was slightly colored. The solvent was removed by blowing with nitrogen and 0.5% to 1% solutions of the methyl esters were prepared in acetone. Two-tenths to 2 μl of these solutions were injected into the column with a Hamilton syringe. Suitable conditions for columns prepared as described were: Column temperature 215° to 220°, flash heater temperature 260° to 290°, detector temperature 235° (radium foil), argon pressure 10 to 15 psi, flow rate 40 to 70 ml/minute.

RESULTS AND DISCUSSION

Table 1 gives the retention times, relative to methyl deoxycholate, of the substituted methyl cholanates tested on two types of column. One type had the silicone rubber with 20 moles per cent phenyl groups as stationary phase, the other one had the polymer with 35 moles per cent phenyl groups. Figures 1 and 2 show the differences in the separating abilities of the two phases.

Methyl deoxycholate was chosen as the reference compound since it has a retention time which is in the middle of the range of retention times found for the various compounds. The conditions were chosen so as to retain methyl deoxycholate for about 20 minutes. Except in the case of the methyl esters of 3α-hydroxy-6-ketocholanic and 3α-hydroxy-6-keto-allocholanic acids (kindly given to us by Dr. E. A. Doisy, Jr., St. Louis University, St. Louis, Mo.), no signs of destruction were noted. These two compounds, however, were apparently partly degraded in the flash heater. The peak shape of keto and acetoxy compounds was symmetrical; di- and trihydroxy compounds showed a slight tailing, which can be prevented by using more polar stationary phases. As seen in Table 1, addition of a hydroxyl group to the cholanic acid nucleus approximately doubles the retention times (see also Table 2).

The isomeric mono- and dihydroxy compounds tested could all be separated (compounds 2, 4, 9, 14, 20), whereas separations of isomeric trihydroxy compounds were unsatisfactory (compounds 22, 23, 33, 34). Also, the stationary phases used did not differentiate an axial and an equatorial hydroxyl at C-7 (compounds 14, 15; and 22, 23), the only position tested in this respect. This is surprising since in reversed phase chromatography such isomeric compounds can be easily separated because of their different polarity. The monoketo compounds (Nos. 3, 6) are retained longer than the corresponding hydroxy compounds. This is also the case with the 3,12-disubstituted compounds (Nos. 9, 11, 13), whereas no change occurs on

TABLE 1. RETENTION TIMES RELATIVE TO METHYL DEOXYCHOLATE OF SUBSTITUTED METHYL CHOLANATES

No.	Compound	Rel. Retention Time 20*	35*	No.	Compound	Rel. Retention Time 20*	35*
1	Methyl cholanate	0.23	0.20	18	3α-acetoxy-7-keto	1.49	1.48
2	12α-hydroxy	0.42	0.38	19	3,7-diketo	1.16	1.21
3	12-keto	0.45	0.42	20	3α,6α-dihydroxy	1.34	1.30
4	3α-hydroxy	0.54	0.49	21	3α,6α-diacetoxy	1.92	1.72
5	3α-acetoxy	0.79	0.67	22	3α,7α,12α-trihydroxy	2.20	2.32
6	3-keto	0.62	0.58	23	3α,7β,12α-trihydroxy	2.30	2.36
7	3α-hydroxy-11-ene	0.53	0.48	24	3α,7α-diacetoxy-12-hydroxy	2.44	2.44
8	3β-hydroxy-5-ene	0.64	0.58	25	3α,7α,12α-triacetoxy	1.88	1.90
9	3α,12α-dihydroxy	1.00	1.00	26	3α,12α-dihydroxy-7-keto	2.20	2.36
10	3α,12α-diacetoxy	1.15	1.14	27	3α,12α-diacetoxy-7-keto	2.24	2.27
11	3α-hydroxy-12-keto	1.15	1.12	28	3α,7α-dihydroxy-12-keto	2.62	2.73
12	3α-acetoxy-12-keto	1.46	1.43	29	3α,7α-diacetoxy-12-keto	2.57	2.59
13	3,12-diketo	1.27	1.25	30	3α-hydroxy-7,12-diketo	2.18	2.34
14	3α,7α-dihydroxy	1.16	1.18	31	3α-acetoxy-7,12-diketo	2.52	2.72
15	3α,7β-dihydroxy	1.12	1.16	32	3,7,12-triketo	2.03	2.30
16	3α,7α-diacetoxy	1.35	1.29	33	3α,6α,7α-trihydroxy	2.41	2.62
17	3α-hydroxy-7-keto	1.14	1.21	34	3α,6β,7β-trihydroxy	2.20	2.24

* Mole per cent phenyl groups in the stationary phase.

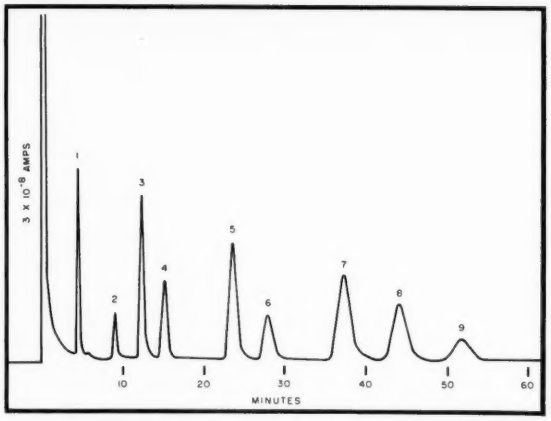

FIG. 1. Five-tenths per cent SE-52 silicone gum rubber, 20 moles per cent phenyl groups, on Gas-Chrom P, 100 to 120 mesh. Peak *1:* cholanic; peak *2:* 12-keto; peak *3:* 3-keto; peak *4:* 3α-acetoxy; peak *5:* 3α-12α-diacetoxy; peak *6:* 3α,7α-diacetoxy; peak *7:* 3α,7α,12α-triacetoxy and 3α,6α-diacetoxy; peak *8:* 3α,12α-diacetoxy-7-keto; peak *9:* 3α-acetoxy-7,12-diketo.

oxidation of the 3,7-dihydroxy compound to the ketones (compounds 14, 17, 19). Similarly, oxidation of the 7α-hydroxyl group in methyl cholate (No. 22) does not change the retention time (No. 26), whereas the corresponding change from 12α-hydroxy to 12-keto (No. 28) increases the retention time.

Several compounds with identical retention times can be separated after acetylation. Generally acetylation increases the retention time of mono- and disubstituted compounds, whereas for trisubstituted ones the effect is small and in some cases the retention time is decreased (compounds 22, 25; and 28, 29). Introduction of a 3α-carbethoxy group in deoxycholic acid more than doubled the retention time.

The number of CH_2-groups in the side chain has a pronounced effect on the retention time, as shown in Table 3. Comparison between compounds 37 and 41 shows that conversion of the C-27 methyl group in trihydroxy coprostane into the methyl ester group of methyl trihydroxy coprostanate increases the retention time about threefold.

The percentage of phenyl groups in the stationary phase influences the retention times of the compounds such that on the phase with the higher percentage of phenyl groups the hydroxyl and keto groups on the compounds cause a longer retention time (Table 1). Acetylation of hydroxyl groups results in a smaller relative retention time on the 35% column than on the 20% column.

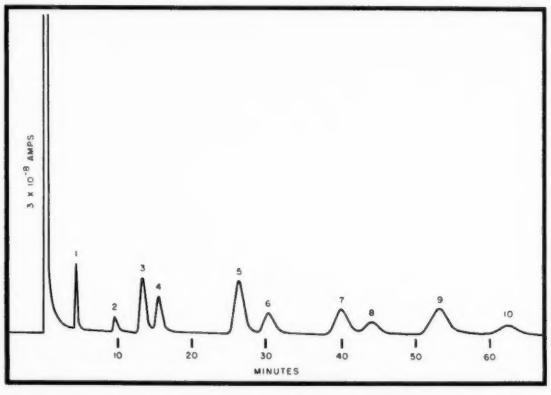

FIG. 2. Five-tenths per cent SE-52 silicone gum rubber, 35 moles per cent phenyl groups, on Gas-Chrom P, 100 to 120 mesh. Peak *1:* cholanic; peak *2:* 12-keto; peak *3:* 3-keto; peak *4:* 3α-acetoxy; peak *5:* 3α,12α-diacetoxy; peak *6:* 3α,7α-diacetoxy; peak *7:* 3α,6α-diacetoxy; peak *8:* 3α,7α,12α-triacetoxy; peak *9:* 3α,12α-diacetoxy-7-keto; peak *10:* 3α-acetoxy-7,12-diketo.

TABLE 2. RETENTION TIMES RELATIVE TO METHYL DEOXYCHOLATE OF MONO-, DI-, AND TRIHYDROXY COPROSTANES RELATED TO BILE ACIDS

No.	Compound	Rel. Retention Time 20*	35*
35	3α-hydroxy coprostane	0.36	0.27
36	3α,12α-dihydroxy coprostane	0.67	0.56
37	3α,7α,12α-trihydroxy coprostane	1.42	1.29

* Mole per cent phenyl groups in the stationary phase.

TABLE 3. EFFECT OF SIDE CHAIN LENGTH ON RELATIVE RETENTION TIME

No.	Compound	Rel. Retention Time 20*	35*
38	Methyl 3α,12α-diacetoxy bisnorcholanate	0.55	0.51
39	Methyl 3α,12α-diacetoxy norcholanate	0.86	0.82
10	Methyl 3α,12α-diacetoxy cholanate	1.15	1.14
40	Methyl 3α,7α,12α-trihydroxy bisnorcholanate	1.03	1.17
22	Methyl 3α,7α,12α-trihydroxy cholanate	2.20	2.32
41	Methyl 3α,7α,12α-trihydroxy coprostanate	4.37	4.23
37	3α,7α,12α-trihydroxy coprostane	1.42	1.29

* Mole per cent phenyl groups in the stationary phase.

Although it is not possible with the phases described in this paper to separate all possible fecal bile acids, the methods described should be of value for the tentative identification or quantitative determination of bile acids, particularly if the sample is analyzed before and after acetylation or oxidation.

REFERENCES

1. Bergström, S., and J. Sjövall. *Acta Chem. Scand.* **5:** 1267, 1951.
2. Sjövall, J. *Acta Physiol. Scand.* **29:** 232, 1953.
3. Norman, A. *Acta Chem. Scand.* **7:** 1413, 1953.
4. Mosbach, E. H., C. Zomzely and F. E. Kendall. *Arch. Biochem. Biophys.* **48:** 95, 1954.
5. Sjövall, J. *Acta Chem. Scand.* **8:** 339, 1954.
6. Sjövall, J. *Clin. Chim. Acta* **4:** 652, 1959.
7. Ahrens, E. H., Jr., and L. C. Craig. *J. Biol. Chem.* **195:** 763, 1952.
8. Wootton, I. D. P. *Biochem. J.* **53:** 85, 1953.
9. Hamilton, J. G., and J. W. Dieckert. *Arch. Biochem. Biophys.* **82:** 203, 1959.
10. Vanden Heuvel, W. J. A., C. C. Sweeley and E. C. Horning. *Biochem. Biophys. Research Commun.* **3:** 33, 1960.
11. Horning, E. C., E. A. Moscatelli and C. C. Sweeley. *Chem. & Ind. (London)*, 751 (1959).

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The isolation and characterization of phospholipids containing mono- and dimethylethanolamine from *Neurospora crassa**

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SUMMARY

Mono- and dimethylethanolamine containing phospholipids have been isolated from a choline-requiring mutant strain of *Neurospora crassa*. These phospholipids have been chemically degraded, and shown to be the phosphatidyl esters of mono- and dimethylethanolamine. The implications of the accumulation of these compounds by the mutant of *Neurospora* are discussed.

We have previously reported the isolation of MMEA[1] and DMEA from the lipid hydrolyzate of *Neurospora crassa*, strain 47904 (1). The chromatographic behavior of these metabolites indicated that they were the phosphatidyl derivatives of MMEA and DMEA. The isolation, from 47904, of the corresponding free amines and also the phosphate esters of these two bases (2) suggests two alternate pathways for the synthesis of phosphatidyl-MMEA, phosphatidyl-DMEA, and lecithin: (*a*) Kennedy's scheme (3) via the cytidine diphosphate base, and (*b*) a direct methylation of cephalin. This latter proposal is supported by the work of Bremer and Greenberg (4, 5), and that of Artom and Lofland (6, 7).

This paper is concerned with the separation and chemical characterization of the phospholipids from "wild-type" *Neurospora*, strain 1A, and from the mutant strain, 47904. A quantitative estimate of the amounts of MMEA, DMEA, and choline liberated on hydrolysis of the phospholipids from strain 47904 has been published previously (1).

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[1] The following abbreviations are used: MMEA, monomethylethanolamine; DMEA, dimethylethanolamine; MMEA-P, phosphorylmonomethylethanolamine; DMEA-P, phosphoryldimethylethanolamine; GPM, glycerophosphorylmonomethylethanolamine; GPD, glycerophosphoryldimethylethanolamine; GPC, glycerophosphorylcholine.

EXPERIMENTAL

Cultures. *Neurospora crassa*, strain 47904, previously described by Horowitz *et al.* (8, 9) synthesizes MMEA and DMEA (10), but is unable to convert these compounds to choline at the normal rate, due to a mutation at a genetic locus concerned with the synthesis of choline. This mutant has also been shown to accumulate both MMEA-P and DMEA-P (2). Large quantities of these four compounds accumulate in the mycelium of a 47904 culture grown under forced aeration.

The "wild-type" *Neurospora*, strain 1A, by comparison, accumulates large quantities of choline and phosphorylcholine, no MMEA or MMEA-P, and trace amounts of DMEA and DMEA-P.

The quantitative estimation of the free bases, MMEA, DMEA, and choline, as well as the bases liberated from phosphate esters after hydrolysis, is determined with another choline-requiring mutant, *Neurospora crassa*, strain 34486 (11).

Growth and Extraction of Microorganisms. In each experiment a 6-liter culture of *Neurospora crassa*, strain 47904 or 1A, is grown for 6 or 10 days at 25° under forced aeration on minimal medium (12). The mycelium is homogenized with distilled water in a Waring Blendor and filtered. Trace amounts of lipids are recovered from the filtrate by extraction with chloroform before the filtrate is discarded. In earlier studies (1), the total lipids were removed from the homogenized mold by extraction with ether and alcohol; however, in later experiments low temperature chloroform extractions

according to Wren and Mitchell (13) were employed. The total lipid recovered from either strain 1A or 47904 corresponds to 10 ± 1% of the dry weight of the mycelium. Essentially the same results are obtained from studies on phospholipids obtained by both extraction methods. These lipid extracts are washed free of nonlipid contaminants by the method of Folch *et al.* (14). Since the phospholipids in peaks *A* and *B* (Fig. 1) are readily oxidized, all procedures concerned with these materials were designed to minimize exposure of these lipids to air.

Silicic Acid. The chromatographic separation of total lipids on a silicic-acid column is based on a solvent system previously employed for blood lipids by Mead and Fillerup (15), while the separation of the phospholipids on silicic acid is carried out according to the method of Wren and Mitchell (13).

Silicic acid (Baker analyzed) is prepared essentially according to the method described by Hirsch and Ahrens (16), except that it is used without being ground in an Abbé mill. Fifty grams of the washed and dried silicic acid is dusted into a column (2.8 cm diameter) provided with a sintered glass plug at its lower end, which is covered with a piece of thick filter paper. The silicic acid is packed to a column height of about 11.8 cm by applying suction to the lower end of the column and tapping the sides of the glass. A thick filter paper disk is pressed firmly on the top of the silicic acid to minimize distortion of the upper surface during elution.

The silicic-acid column described above is washed with 100-ml aliquots of methanol, acetone, and diethyl ether, respectively. The sample is put on the column in diethyl ether, and the nonpolar lipids are removed by passing through 500 ml of ether. The phospholipids are eluted from the column with 500 ml of methanol. The phospholipids recovered from either strain 1A or 47904 represent about 3.5% to 4% of the dry weight of the mycelium. A comparison of the phospholipids from these two strains of *Neurospora*, based on the methylated ethanolamine content, was made in a previous publication (1).

For the separation of phospholipids, a silicic-acid column packed identically to that described above is washed with 400 ml of chloroform. The methanol eluate from the previously described silicic-acid column is evaporated to dryness and the lipid residue applied to the column in 5% methanol in chloroform (v/v). The column is developed with increasing percentages of methanol in chloroform at a rate of flow set at 14 to 16 ml/hour. Samples are collected at half-hour intervals. The test tubes in which the samples are collected are covered with funnels during collection to prevent evaporation. For phosphorus analyses, aliquots of each tube are taken to dryness and determined according to the method of Bartlett (17). Samples are prepared for the methylated ethanolamine analysis of each eluate fraction by taking aliquots to dryness and subsequently hydrolyzing each residue by autoclaving

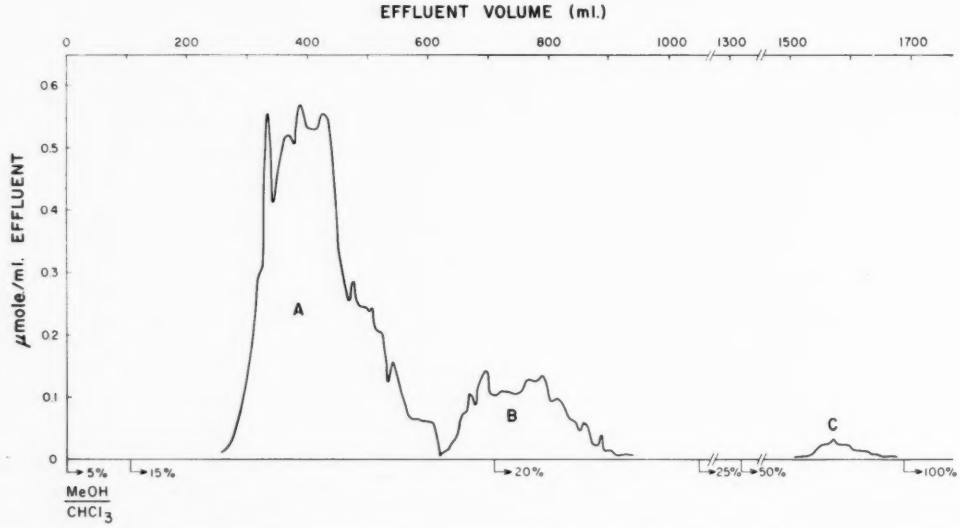

FIG. 1. Silicic-acid chromatography of phospholipids extracted from *Neurospora crassa*, strain 47904. The column was charged with 675 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced. The values on the ordinate are expressed as micromoles of MMEA (peak *A*), DMEA (peak *B*), and choline (peak *C*) per milliliter of eluate.

for 6 hours with 3% sulfuric acid at 250°F. The cooled hydrolyzates are extracted with chloroform to remove any lipid contaminants. The MMEA, DMEA, and choline in the aqueous phase are assayed as previously described (11). Figures 1 and 2 show the results of a typical separation. The results are expressed as micromoles of MMEA, DMEA, or choline per milliliter of eluate. Phospholipids from both 6- and 10-day-old cultures gave essentially the same pattern.

Ion-Exchange Resins. The separation of free MMEA, DMEA, and choline on a Dowex 50 cation-exchange column (15 × 450 mm, hydrogen form) has been described previously (10). The choline chloride used in the present studies was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Monomethylethanolamine, ootained from Eli Lilly and Company, Indianapolis, Indiana, was redistilled in a 28-inch Vigreaux fractionating column, and the fraction boiling at 155°–155.5° was used in these studies. DMEA (Eastman-Kodak Company, Rochester, New York) was redistilled in a similar column. A fraction boiling at 58°–60° at 50-mm pressure was employed as an authentic reference sample.

Both MMEA-P and DMEA-P are products obtained by the chemical degradation of the phospholipids found in *Neurospora*, strain 47904. These esters are identified by comparing their chromatographic behavior with authentic samples on both a Dowex 50 cation-exchange column (15 × 450 mm, hydrogen form) (2) as well as on a Dowex 1 × 4 anion-exchange column (15 × 450 mm, acetate form). Upon elution of the above anion-exchange column with 200 ml of water, followed by 0.1 N acetic acid, authentic MMEA-P and DMEA-P both emerge from such a column with concentration peaks, as determined by bioassay, at 295 to 305 ml of total eluate. Chromatography on the Dowex 50 cation-exchange column with 0.3 N HCl as the eluting solvent gives individual peaks for MMEA-P and DMEA-P with maxima at 340 ml and 407 ml, respectively. These values differ slightly from the previously published values for MMEA-P and DMEA-P (2). The Dowex 50 resin used in these experiments was obtained from J. T. Baker Chemical Company, Phillipsburg, New Jersey. The authentic MMEA-P and DMEA-P employed in the present studies were those described in a previous publication (2).

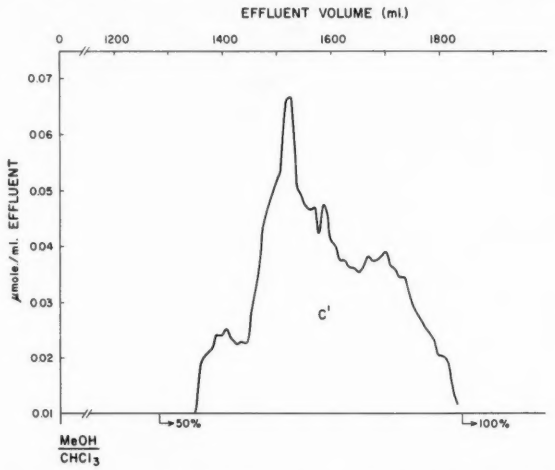

FIG. 2. Silicic-acid chromatography of phospholipids extracted from *Neurospora crassa*, strain 1A. The column was charged with 101 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced. Solvent changes between 0 and 1200 ml were the same as in Figure 1. The values on the ordinate are expressed as micromoles of choline per milliliter of eluate.

Degradation of Phosphatidyl MMEA and DMEA. The phospholipids contained in peaks *A* and *B* (Fig. 1) are subjected to mild alkaline hydrolysis according to the method of Dawson (18). The neutralized hydrolyzate is partitioned between chloroform-methanol and water by the method of Folch *et al.* (14). The organic phase contains the fatty acids which result from the hydrolysis of the phospholipids. These are recovered and stored under nitrogen for analysis by gas-liquid chromatography. The aqueous phase contains the glycerophosphoryl esters of the two methylated ethanolamines. The glycerol moiety is removed from these products of hydrolysis to yield the phosphate esters of MMEA and DMEA by the method of Fleury *et al.* (19) as follows: A slight excess of periodate is allowed to react with the phosphate diesters for 30 minutes. The periodate and iodate ions present at the completion of the oxidation are removed as the insoluble barium salts by the addition of barium carbonate. The filtrate is taken to dryness at 25° and dissolved in a small volume of 1 N sodium acetate buffer at pH 4. A stoichiometric amount of phenylhydrazine is added, based on the amount of periodate previously consumed by the sample. The latter values are obtained on small aliquots of the periodate reaction mixture by back titration of unused periodate. The buffered reaction mixture is kept at 37° for 1 hour, after which it is extracted several times with 5-ml volumes of ether. The aqueous phase contains the desired phosphate esters of MMEA and DMEA, which are then characterized by comparing their behavior on ion-exchange resin columns with authentic materials.

RESULTS

Identification of MMEA, DMEA, and Choline. MMEA, DMEA, and choline were obtained with C^{14}

in their methyl groups from the completely hydrolyzed phospholipids of either culture 1A or 47904, which had been grown on isotopically labeled formate-C^{14}. The isolation of these amines on a Dowex 50 column with 1.5 N HCl as the eluting solvent has been described in previous communications (10, 20). Each of the isolated labeled amines was isotopically diluted with authentic unlabeled material, and the resulting mixture rechromatographed on a second Dowex 50 column. In each case the biological activity, as measured by bioassay with *Neurospora crassa*, strain 34486, is superimposed upon the radioactivity in the chromatographic patterns, which are plotted for eluate fractions. Further confirmation of the identity of the isolated three amines was obtained by comparing the growth response of *Neurospora crassa*, strain 34486, to each isolated substance, with simultaneous assays on authentic material. In each case the growth response of the organism to increasing aliquots of the isolated amines in question corresponds to the growth curves obtained on authentic samples. The crystalline picrate of MMEA (m.p. 148°–150°) was isolated from the phospholipid hydrolyzate of two combined 6-liter cultures of strain 47904 grown for 10 days. This sample does not exhibit a melting point depression when mixed with an authentic sample (m.p. 148°–149.5°).

Each of the fractions *A*, *B*, *C*, and *C′* indicated in Figures 1 and 2 were hydrolyzed according to the method of Horowitz and Beadle (11). The hydrolyzates were chromatographed on Dowex 50 columns to determine the distribution of the methylated ethanolamines contained within each fraction, the eluates being assayed by means of *Neurospora crassa*, strain 34486. These experiments have shown that fraction *A* contains only MMEA, fraction *B* contains only DMEA, and fractions *C* and *C′* both yield only choline. Hence there is a clear separation of phospholipids based on amine content as well as chromatographic behavior. Since peaks *A* and *B* (Fig. 1) almost completely replace the lecithin found in the normal strain (Fig. 2), further studies on the chemical characterization of these materials were based on the conjecture that these peaks contained the phosphatidyl esters of MMEA and DMEA. In the work discussed in the following section, GPM was studied more extensively than GPD since much larger quantities of the former were available.

Characterization of MMEA-P, DMEA-P, and GPC. When known chemical degradative methods previously used for studying the chemistry of lecithin and cephalin are applied to the two unknown phospholipid fractions *A* and *B* (Fig. 1), the products obtained are those normally expected from a phosphatidyl derivative. Mild alkaline hydrolysis of all of the phospholipid fractions (18) yields fatty acids which are readily extractable from an aqueous phase with chloroform. The aqueous phases from the hydrolyzates, remaining after the release and extraction of fatty acids with organic solvent, contained the biologically active amines in ester forms, presumably as the glycerophosphoryl bases. Since the expected water-soluble hydrolysis product of fractions *C* and *C′* is a known and available substance previously described in the literature, its identity is readily confirmed by comparing its chromatographic behavior on a column of powdered cellulose (15 × 450 mm) with an authentic sample. The eluting solvent in this study is a mixture consisting of eight parts of 99% ethyl alcohol and one part of 0.1 N acetic acid-pyridine buffer, pH 4.5.[2] The elution peak of the unknown sample is between 243 to 254 ml of the eluting solvent, and is indistinguishable from the behavior of authentic GPC. Since the expected corresponding hydrolysis products from peaks *A* and *B*, GPM and GPD, respectively, are chemically unknown, the identification of these materials by direct comparison with authentic materials is impossible at this time. An unequivocal identification of these degradation intermediates by classic chemical approaches will be attempted when adequate amounts of pure materials are available. Work directed toward this goal has been slow.

Before proceeding further with the degradation of these materials, a preliminary experiment was performed on GPM to determine whether it is oxidized by periodate, and whether its chromatographic behavior on ion-exchange resins resembles that of GPC, which has a similar dipolar-ion structure. A phosphate diester such as GPC passes readily through anion-exchange resins without expressing an appreciable anionic character. The substance believed to be GPM also exhibits this predicted behavior and passes through chromatographic columns of Dowex 1 × 4 at the solvent front when distilled water is employed as the eluting solvent. An aliquot of GPM used up 10% more than the theoretical amount of periodate according to the method of Voris *et al.* (21).

The biologically active substances resulting from the mild alkaline hydrolysis of fractions *A* and *B*, presumably GPM and GPD, were degraded to MMEA-P and DMEA-P according to the method of Fleury *et al.* (19), by a procedure previously applied to the degradation of authentic GPC[3] and glycerophosphorylinositol (22). Glycolaldehydephosphorylcholine, obtained by

[2] B. J. Crocken and J. F. Nyc. Unpublished results.
[3] M. O. Hall and J. F. Nyc. Unpublished results.

treating GPC with periodate, spontaneously decomposes to yield phosphorylcholine when allowed to react with phenylhydrazine at 37°. By an analogous procedure, Brown *et al.* (22) obtained phosphorylinositol from glycerophosphorylinositol. The MMEA and DMEA phosphate esters, which were obtained by this degradation method, were characterized by comparing their behavior with authentic samples on both the Dowex 1 anion- and the Dowex 50 cation-exchange columns described above. The elution pattern of these phosphate esters was indistinguishable from that of the authentic controls in these chromatographic studies.

Characterization of Phospholipids. When a hydrolyzed sample of total phospholipid from strain 47904 is subjected to paper chromatography according to Bremer and Greenberg (4), it is found to contain MMEA, DMEA, choline, ethanolamine, serine, and two unidentified ninhydrin-staining spots. An inositol test was positive (23). The total phospholipid contains only 0.05 mmole of plasmalogen per gram as determined by the potassium tri-iodide method of Rapport and Alonzo (24).

About one-third of the total phospholipid from strain 47904 is recovered as peak *A* (Fig. 1). A typical sample contains 0.7 mmole of MMEA and 1.2 mmoles of phosphorus (17) per gram of material. The low iodine uptake of 0.014 mmole per gram of this lipid indicates that about 1% of the phosphorus in the sample is accounted for by plasmalogens. When a hydrolyzate of peak *A* is chromatographed on paper (4), the ninhydrin-staining contaminants are ethanolamine (estimated at 0.05 mmole per gram of lipid on the basis of the ninhydrin color reaction) and traces of serine. The sample does not contain detectable amounts of inositol. A surprising observation is the fact that about a half of the MMEA-containing material in crude phospholipid extracts of strain 47904 is not eluted from a silicic-acid column by 50% methanol-chloroform. The phosphatidyl-MMEA in peak *A* can be rechromatographed on a silicic-acid column without appreciable loss of material. It is not known at present whether the unidentified MMEA-containing material is an artifact resulting from the isolation procedure.

To further purify the phosphatidyl-MMEA in peak *A*, the tubes comprising this fraction were combined, evaporated to dryness, and the resulting residue was rechromatographed on silicic acid under conditions identical to those employed for the experiment illustrated in Figure 1. As can be seen in Figure 3, a separation into two phosphorus-containing peaks, A^1 and A^2, is accomplished. Peak A^1, which contains all of the MMEA, gives a molar ratio of MMEA to phosphorus of 0.92. There is no measurable loss of

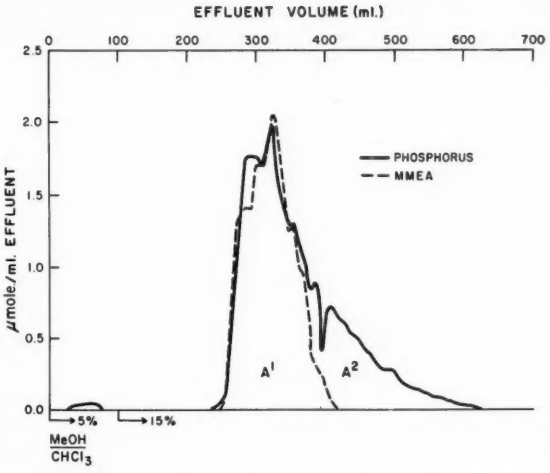

FIG. 3. Rechromatography of peak *A* on silicic acid. The column was charged with 227 mg of phospholipid. Marks on the abscissa show when changed solvents were introduced.

the MMEA moiety during the rechromatography of peak *A*, and the total recovery of phosphorus in the fractions corresponding to peaks A^1 and A^2 is 92% of the amount originally contained in the sample subjected to rechromatography. Studies employing paper chromatography revealed that most of the ethanolamine and all of the serine, which were previously present in hydrolyzates of peak *A*, are now in peak A^2. This fraction contains 26% of the total phosphorus recovered after the rechromatography of peak *A*.

One gram of the total lipid from strain 47904 yields 150 mg of phospholipid in the eluate fraction corresponding to peak *B* (Fig. 1). Essentially all of the DMEA-containing lipid in the total lipid fraction is recovered in the material contained in peak *B*. A typical sample contains 0.7 mmole of DMEA and 1.3 mmoles of phosphorus per gram of material. When a hydrolyzate of the lipid in peak *B* is chromatographed on paper, the only ninhydrin-staining materials which are observed are ethanolamine and serine. Estimates based on such studies indicate that the phosphatidyl derivatives of ethanolamine and serine are the major contaminants of phosphatidyl-DMEA. Inositol-containing phospholipids are not found in this fraction. The iodine uptake of 0.02 mmole per gram of lipid shows that the plasmalogens represent only a small fraction of the lipids in peak *B*. Further purification of phosphatidyl-DMEA has not been effected at this time.

DISCUSSION

The isolation of phospholipids containing MMEA

and DMEA from a *Neurospora* mutant grown only on sugar, salts, and biotin establishes these substances as naturally occurring products of cell metabolism (1). These phospholipids have now been identified as the phosphatidyl esters of the corresponding methylated ethanolamines. The natural occurrence of these lipids suggests that they play a metabolic role as precursors of lecithin, and thus may be obligatory intermediates in the *de novo* synthesis of a choline moiety. The studies with *Neurospora* parallel studies with mammalian tissues in two other laboratories. Bremer and Greenberg (4, 5) have shown that rat liver microsomes incubated with (Me-C^{14})-adenosylmethionine were able to incorporate the label into phospholipids from which radioactive MMEA and DMEA could be isolated. In these studies it was noted that the maximum incorporation of radioactivity into cytidinediphosphocholine occurred 1 to 2 hours later than that in the phospholipid choline. More recently, Artom and Lofland (6, 7) have incubated enzymatically prepared phosphatidyl-DMEA-C^{14} with a rat liver homogenate, and found that unless adenosylmethionine is added to the incubation mixture, no label is found in lecithin. No isotope dilution effect is noted when unlabeled DMEA is added to the incubation flasks. This indicates that DMEA is not split off the phospholipid moiety before methylation, but rather that the methylation occurs on the intact phospholipid. The above workers (4 to 7) believe that the methylation of phosphatidyl ethanolamine or phosphatidyl-DMEA represents an important pathway in the biosynthesis of lecithin.

Since the same mutant of *Neurospora* in which these MMEA- and DMEA-containing phospholipids are found also accumulates free MMEA, DMEA (10), and the phosphate esters of these two bases (2), an important consideration arises concerning the precursor-product relationship of these bases and base esters to the corresponding phosphatidyl derivatives. Mycelial extracts of 6- and 10-day-old *Neurospora* 47904 cultures both contain essentially the same concentration of the phosphatidyl esters of MMEA, DMEA, and choline. In contrast to this, earlier work by Horowitz (9) showed that free MMEA did not appear in the medium of a liquid culture of this organism until after the sixth day of growth—at which time appreciable autolysis can occur. He used *Neurospora*, strain 34486, to quantitate the amount of base in the media of cultures of strain 47904. Because strain 34486 responds to MMEA, DMEA, and choline, he would have detected the presence of any one of these bases in his time-accumulation studies. Since the free methylated ethanolamines appear only in the media of aging 47904 cultures, they may be, to some extent, autolysis products formed by the degradation of the corresponding phospholipids. Artom *et al.* (7, 25) have shown that in rat liver slices DMEA can be incorporated into a phospholipid, presumably by a pathway analogous to that proposed by Kennedy (3) for the biosynthesis of phosphatidyl ethanolamine and lecithin. The proposed degradation of MMEA- and DMEA-containing phospholipids in autolyzing cultures of *Neurospora* may represent a reversal of Kennedy's synthetic pathway. The DMEA-containing phospholipid prepared by Artom and Lofland (7) was eluted from a silicic-acid column by a 4 to 1 chloroform-methanol eluant. This corresponds well with the behavior of the phosphatidyl-DMEA in peak *B* (Fig. 1), which was obtained from *Neurospora*.

It is well established that serine can give rise to ethanolamine by decarboxylation. In view of the experiments reported here, and those by Bremer and Greenberg (4, 5) and Artom and Lofland (6, 7), it is interesting to speculate that a phosphatidyl serine decarboxylase might exist. This could catalyze the decarboxylation of phosphatidyl serine to phosphatidyl ethanolamine, which would then be the initial methyl acceptor for lecithin synthesis. It is noteworthy in this connection that Hübscher *et al.* (26) have shown that the biosynthetic pathway of phosphatidyl serine differs from that of phosphatidyl choline or phosphatidyl ethanolamine.

Preliminary experiments have shown a large difference in the fatty acid composition of the various phospholipids from strains 1A and 47904. Further purification of the phospholipids from peaks *A*, *B*, *C*, and *C'* is necessary before any conclusions can be drawn from these results. Work directed toward this goal is in progress in this laboratory.

The single-gene mutant of *Neurospora*, strain 47904, lacking choline, differs phenotypically from the normal "wild-type" strain 1A only in its reduced rate of growth (8), greater aminopterin resistance (27), and colonial form of growth on liquid medium. Despite the fact that the mutant strain 47904 lacks most of the lecithin normally found in other strains, it exhibits a normal vegetative and sexual life cycle (20).

Of interest is the observation (28) that the *tubercle bacillus*, like strain 47904, also lacks lecithin. It is not known at this time whether this organism has substituted MMEA- and DMEA-containing phospholipids for the lecithin which it lacks.

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REFERENCES

1. Hall, M. O., and J. F. Nyc. *J. Am. Chem. Soc.* **81:** 2275, 1959.
2. Wolf, B., and J. F. Nyc. *J. Biol. Chem.* **234:** 1068, 1959.
3. Kennedy, E. P. *Ann. Rev. Biochem.* **26:** 119, 1957.
4. Bremer, J., and D. M. Greenberg. *Biochim. et Biophys. Acta* **35:** 287, 1959.
5. Bremer, J., and D. M. Greenberg. *Biochim. et Biophys. Acta* **37:** 173, 1960.
6. Artom, C. *Federation Proc.* **19:** 233, 1960.
7. Artom, C., and H. B. Lofland, Jr. *Biochem. Biophys. Research Commun.* **3:** 244, 1960.
8. Horowitz, N. H., D. Bonner and M. B. Houlahan. *J. Biol. Chem.* **159:** 145, 1945.
9. Horowitz, N. H. *J. Biol. Chem.* **162:** 413, 1946.
10. Wolf, B., and J. F. Nyc. *Biochim. et Biophys. Acta* **31:** 208, 1959.
11. Horowitz, N. H., and G. W. Beadle. *J. Biol. Chem.* **150:** 325, 1943.
12. Beadle, G. W., and E. L. Tatum. *Am. J. Botany* **32:** 678, 1945.
13. Wren, J. J., and H. K. Mitchell. *J. Biol. Chem.* **234:** 2823, 1959.
14. Folch, J., I. Ascoli, M. Lees, J. A. Meath, and F. N. LeBaron. *J. Biol. Chem.* **191:** 833, 1951.
15. Mead, J. F., and D. L. Fillerup. *J. Biol. Chem.* **227:** 1009, 1957.
16. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233:** 311, 1958.
17. Bartlett, G. R. *J. Biol. Chem.* **234:** 466, 1959.
18. Dawson, R. M. C. *Biochim. et Biophys. Acta* **14:** 374, 1954.
19. Fleury, P., J. Courtois and A. Desjobert. *Bull. soc. chim. France* **19:** 458, 1952.
20. Nyc, J. F. *J. Biol. Chem.* **223:** 811, 1956.
21. Voris, L., G. Ellis and L. A. Maynard. *J. Biol. Chem.* **133:** 491, 1940.
22. Brown, D. M., G. E. Hall and R. Letters. *J. Chem. Soc.* Part VI, 3547 (1959).
23. Feigl, F. *Spot Tests in Organic Analysis.* New York, Elsevier Publishing Co., 1956, p. 393.
24. Rapport, M. M., and N. F. Alonzo. *J. Biol. Chem.* **235:** 1953, 1960.
25. Artom, C., and M. Crowder. *Eighteenth International Physiological Congress*, Copenhagen, 1950, p. 78.
26. Hübscher, G., R. R. Dils and W. F. R. Pover. *Nature* **182:** 1806, 1958.
27. Swenseid, M. E., and J. F. Nyc. *J. Bacteriol.* **75:** 654, 1958.
28. Hoshino, N. *J. Biochem. (Tokyo)* **46:** 825, 1959.

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The nitrogenous constituents of the lipids of several dog tissues*

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SUMMARY

A technique is described for the resolution and quantitative determination of the nitrogenous constituents of dog tissue lipids. Results of representative analyses are presented for seven different tissues. All but a few per cent of the total lipid nitrogen has been specifically accounted for as choline, ethanolamine, serine, other amino acids, ammonia, sphingosine, and hexosamine. Serine-containing lipids comprise a maximum of 2.6% to 7.7% of total lipid nitrogen of the tissues studied. Despite extensive purification of the lipid extracts, other amino acids and peptides are present in quantities comparable to serine. The ammonia content of the lipid hydrolyzates ranges from 4.3% to 5.9% of lipid nitrogen. From 1.0% to 9.4% of lipid nitrogen becomes water soluble after hydrolysis, and is neither choline, ammonia, nor primary amine. This is considered to be substituted amine, although no important fraction of this material has been identified.

A number of analytical procedures have been used for the determination of the nitrogenous components of lipids (1, 2, 3). However, a complete analysis of the lipids of animal tissues for all the components is beset with several difficulties. First, lipids may combine with amino acids and other nitrogenous substances to form rather stable, solvent-soluble adducts as an artifact of extraction (4, 5). The amount of protein or peptide in the lipid extracts depends on the extraction procedure (6, 7) and the method of purification. Some of this peptide may be split from the lipids by further treatment with neutral solvents, and is therefore not a true component of the lipids. Second, amino acids and peptides interfere with most of the primary amine reactions which have been used to measure ethanolamine and serine. Much of the existing data on the distribution of these substances is open to question. Third, the stability of certain lipids to hydrolytic agents (8) necessitates drastic conditions of hydrolysis which may destroy the liberated bases or produce artifacts. Finally, purified animal tissue lipids contain minor amounts of unidentified nitrogenous substances (9).

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This paper describes the fractionation of the hydrolysis products and a reasonably complete analysis of the fractions. The procedure includes (*a*) removal of most of the interfering peptide, (*b*) a two-stage hydrolysis which permits removal of the free bases released by mild hydrolytic conditions before the use of more drastic conditions, (*c*) a chromatographic resolution of the base mixture for a more accurate determination of the minor components, and (*d*) the use of periodate oxidation, methylation, and paper chromatography for a specific analysis of the primary amines. The results of analyses of a variety of dog tissues are presented and compared with those from other laboratories.

EXPERIMENTAL

Extraction and Purification. Frozen tissue powder was refluxed with ethanol-ether 3/1 (v/v) for $1^1/_2$ hours, as described previously (10). The dried tissue residue was then extracted with chloroform, in a Soxhlet extractor, and the two extracts were combined. The extracts were purified by re-extraction with chloroform and emulsification into 0.25 M $MgCl_2$, as described (10). However, the final emulsion interphase was not combined with the clear chloroform infranatant. Instead, it was dissolved in a minimal volume of

chloroform-methanol 2/1 (v/v), and the resulting upper aqueous-alcohol phase was discarded. The upper phase contained insignificant amounts of phospholipid and sphingolipid but much peptide material, as evidenced by its nitrogen content (14% to 15%) and the release of 70% to 90% of its nitrogen as free amino nitrogen by acid hydrolysis. The lower chloroform phase was combined with the original washed chloroform infranatant. The lipid extracts gave somewhat lower nitrogen to phosphorus ratios than we have obtained previously for dog tissues and for the same tissues extracted and purified by the method of Folch *et al.* (11). Minimal amounts of "phosphatido-peptides" (7) were present, since neutral solvents were used in the extraction. The extraction of these animal tissues was essentially complete, and the losses of lipid incurred in purification were measured precisely and found to be small (10).

Hydrolysis of the Lipids and Fractionation of the Bases. The over-all procedure is shown diagrammatically in Figure 1. An aliquot of the purified lipids, equivalent to about 3 mmoles of lipid nitrogen, was freed of solvents by vacuum distillation. Eighty milliliters of 1 N NaOH was added, and saponification was allowed to continue at room temperature for 3 days under an acid trap. Fifty-six milliliters of concentrated HCl and then 100 ml of chloroform were added, and the mixture thoroughly shaken. The emulsion was broken by refrigeration and centrifugation, yielding an aqueous phase, fraction A, and a chloroform phase, fraction B.

The aqueous phase, fraction A, was evaporated to dryness under reduced pressure with several additions of water to remove the excess HCl completely. Seventy-five milliliters of absolute ethanol was added, and the mixture was allowed to stand for 24 hours. The alcoholic solution was decanted and the salt residue washed with an additional 35 ml of ethanol, which was then dissolved in the least volume of water (usually about 10 to 15 ml) and precipitated with $1^1/_2$ volumes of alcohol. The salt precipitate was rinsed with alcohol, and the supernatant and washings evaporated to dryness *in vacuo*. The alcohol precipitation from the aqueous solution was repeated twice, and the final alcohol supernatant was added to the original alcohol extracts. The combined salt residues, fraction Al, contained about 1% of the total original lipid nitrogen. The aqueous alcohol extract was evaporated to dryness *in vacuo* and the residue was taken up in 40 ml of water. The solution was adjusted to pH 2 with 1 N NaOH, and any insoluble matter, fraction A2, was centrifuged down. The latter usually contained less than 1% of the original lipid nitrogen and is included in the "Unassigned N" in Table 1. The aqueous solution was then chromatographed on a Dowex 50 Na column prepared according to the method of Moore and Stein (12). The column had an internal diameter of 10 mm and contained 33 cm Dowex. After passage of the solution, the column was washed with 30 ml of 0.01 N HCl, which was combined with the original effluent and designated fraction A3. The column was eluted with 95 ml of 0.05 M sodium acetate buffer, pH 5.0 (fraction A4), 60 ml of 0.05 M $NaHCO_3$–

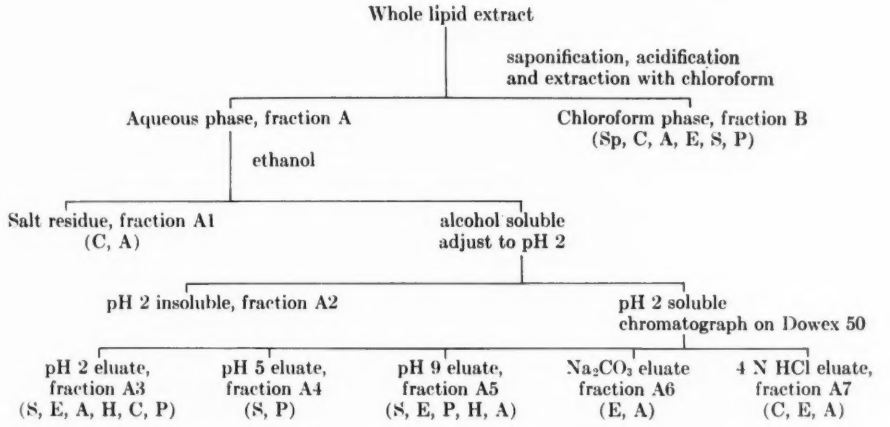
Whole lipid extract
saponification, acidification and extraction with chloroform
Aqueous phase, fraction A
Chloroform phase, fraction B (Sp, C, A, E, S, P)
ethanol
Salt residue, fraction A1 (C, A)
alcohol soluble adjust to pH 2
pH 2 insoluble, fraction A2
pH 2 soluble chromatograph on Dowex 50
pH 2 eluate, fraction A3 (S, E, A, H, C, P)
pH 5 eluate, fraction A4 (S, P)
pH 9 eluate, fraction A5 (S, E, P, H, A)
Na2CO3 eluate fraction A6 (E, A)
4 N HCl eluate, fraction A7 (C, E, A)

FIG. 1. Fractionation scheme for the analysis of lipid extracts. Nitrogenous components determined directly are shown with each fraction in which they occur. *S* = serine, *E* = ethanolamine, *P* = other primary amine, *C* = choline, *Sp* = sphingosine, *H* = hexosamine, *A* = ammonia.

TABLE 1. SUMMARY OF NITROGENOUS COMPONENTS OF DOG TISSUE LIPIDS

Tissue	μmole/g Dry Lipid-Free Tissue		Per Cent of Total Lipid N										
	Total N	Total P	Ethanolamine N	Serine N	Other Primary Amine N	Total Ammonia N	Saponification Ammonia N	Hexosamine N	Choline N	Substituted Amine N		Sphingosine N	Unassigned N
										Total	Fractions A3, A4, and A5 only		
Liver	177	180	17.3	3.4	7.0	4.6	3.9	0.5	49.6	9.4	5.1	4.1	4.5
Spleen	166	136	26.5	7.7	4.6	5.9	4.9	0.5	39.0	4.3	2.6	13.1	0.7
Pancreas	169	173	20.4	2.8	4.1	4.4	4.0	0.2	53.9	3.7	2.8	7.9	3.9
Lung	173	146	20.4	6.6	4.4	4.4	3.3	0.7	45.4	3.3	3.0	13.3	2.4
Lung	167	141	22.6	6.3	3.0	5.0	4.0		44.5	2.4	1.4	9.1	7.7
Kidney	208	188	31.6	4.9	4.1	4.4	3.3	0.3	38.7	3.7	3.4	10.8	4.0
Intestine	119	110	25.9	6.3	4.8	4.8	2.8	0.9	43.0	1.0	0.9	13.7	0.5
Heart	131	148	29.0	2.6	2.5	4.3	3.6	0.4	49.5	5.1	3.8	7.9	1.3

Na_2CO_3 buffer, pH 9.0 (fraction A5), 95 ml of 0.1 M Na_2CO_3 (fraction A6), and finally 35 ml of water, followed by 140 ml of 4 N HCl (fraction A7). These fractions were analyzed by the methods described below.

Analytical Methods. Total nitrogen was determined by a modification of the Kjeldahl method (10). Total phosphorus was determined by a modification of the Fiske and SubbaRow method (10). Ammonia nitrogen was determined by distillation and nesslerization or direct nesslerization (10). Hexosamine was determined by the method of Elson and Morgan (13), as modified by Blix (14), on an aliquot hydrolyzed by refluxing $3^1/_3$ hours with 2 N HCl. Choline was determined as the Reineckate salt (10). Hydrolysis of fraction A3 for choline phosphoric acid was carried out in 5.5 N HCl in sealed tubes at 130° for 15 hours. Primary amines were determined by the naphthoquinone sulfonate color reaction (2). A correction for ammonia content based on the 67% molar color yield given by ammonia was applied, and the corrected value designated "True Amino N" in Table 2. Hydrolysis of fractions A3 and A5 for release of amino nitrogen was carried out in 2 N HCl in sealed tubes at 120° for 15 hours.

Total ethanolamine and serine were determined as ammonia liberated by periodate oxidation under the conditions of Frisell and Mackenzie (15). The ammonia was adsorbed on 1 g of washed Permutit (Folin Decalso) in a 25-ml volumetric flask, washed several times with water to remove formaldehyde, and then eluted directly with Nessler's reagent by the procedure of Folin and Bell (16). The difference between true amino nitrogen and ammonia liberated by periodate is designated "Other Primary Amine" (Table 1). This calculation is valid because the naphthoquinone sulfonate reagent gives approximately the same molar color yield for ethanolamine, serine, other primary amines, and amino acids (17).

The separation of ethanolamine from serine in the hydrolyzate of fraction A3 was carried out by Permutit adsorption, as described previously (2). The Permutit eluates were analyzed for primary amine using the naphthoquinone sulfonate procedure. The "ethanol-

TABLE 2. NITROGENOUS COMPONENTS OF THE AQUEOUS PHASE (FRACTION A) AFTER SAPONIFICATION OF INTESTINE LIPIDS

Fraction	Total N	Total P	Choline N	Ammonia N	True Amino N	Substituted Amine N	Serine N	Ethanolamine N	Other Primary Amine N	Unassigned N
A1	22.7		7.2	1.5	9.8	4.2				9.8
A3	199		0.0	14.7	110					
A3 hydrolyzed			15.9	27.9	148	7.2	44.3	38.7	65.0	0.0
A4	131	0.8	0.0	0.0	125	6.0	88.0	0.0	37	0.0
A5	45.3	0.6	2.4	2.9	12.4					
A5 hydrolyzed				11.2	19.0	12.7			13.4	5.6
A6	558	1.6	0.0	39.4	510	0.0	0.0	523	0.0	0.0
A7	1050	0.0	1040	20.8	18.9	0.0	0.0	18.9	0.0	0.0

All values are given in micromoles. Total original lipid N, 2960 μmoles; P, 2740 μmoles; hexosamine, 27 μmoles.

amine" values may be accepted as ethanolamine, since no basic amino acids are present in the hydrolyzates. In a number of instances, ethanolamine analyses were confirmed by methylation to choline. A suitable aliquot of known choline content was dried *in vacuo* and taken up in 3 to 5 ml of methanol. Methanolic KOH was added until strongly alkaline. Three-tenths of a milliliter of methyl iodide was added, and the mixture was allowed to stand at room temperature overnight. The mixture was acidified with glacial acetic acid, and the solvents removed *in vacuo*. The residue was then taken up in 10 ml of water and choline determined as the reineckate. Ethanolamine is calculated by subtracting the original choline from the total found after methylation. This procedure gives 90% to 95% conversion of ethanolamine, and only 2% to 4% conversion of serine to choline.

Serine is calculated as the difference between total periodate-released ammonia nitrogen and ethanolamine nitrogen. Usually no correction for ammonia is necessary, since this is poorly eluted from Permutit under the conditions of the analysis. There should be no interference from small amounts of N-acylated hexosamine present, since this does not yield ammonia on oxidation with periodate (18).

The "Substituted Amine" in Table 1 is the difference between total Kjeldahl nitrogen of the aqueous phase fractions and the sum of choline, ammonia, and total primary amine.

Paper Chromatography. Three solvent systems were routinely used for ascending chromatograms: (*a*) butanol-glacial acetic acid-water 4:1:5 (v/v), (*b*) butanol-glacial acetic acid-water 2:1:2 (v/v), and (*c*) butanol-ethylene glycol-water 4:1:1 (v/v). In using system (*a*), the paper cylinder was placed in the upper phase, and a beaker containing the lower phase was placed in the middle of the chromatography vessel. Systems (*b*) and (*c*) were monophasic. Fractions A4, A5, A6, and A7 were acidified with HCl, concentrated to dryness under reduced pressure, and extracted with absolute ethanol. After two extractions, the salt residue contained no more than traces of nitrogen. The alcohol extracts were then concentrated and chromatographed. Ninhydrin was used as developer for the primary amines, and Dragendorff's reagent was used for choline.

Analysis of Fraction B. This fraction contained 10.0% to 32.2% of the original lipid nitrogen (Table 3). Only traces of fatty aldehyde were still bound as phospholipid, as revealed by silicic-acid chromatography. Choline and sphingosine were determined on an aqueous $Ba(OH)_2$ hydrolyzate of this fraction. A dried aliquot was refluxed with saturated $Ba(OH)_2$ on a sand bath for 7 hours by the method of McKibbin and Taylor (19). The HCl hydrolysis was omitted to improve recovery of sphingosine (20). For the determination of primary amines, a dried aliquot was refluxed for 12 to 15 hours in 1.6 N ethanolic HCl. The lipids remained in solution throughout the hydrolysis period. The alcohol was evaporated under reduced pressure, and the hydrolyzate was partitioned between chloroform and water Total primary amine, ethanolamine, and serine were determined as described above. The methylation of ethanolamine to choline was especially useful in analyzing for the former in these hydrolyzates. The composition of fraction B from the several tissues is summarized in Table 3. It is seen that ethanolamine contributes a major portion of this nitrogen in all the tissues. The sum of (*a*) the nitrogen of the A fractions, which was not established in the other categories, (*b*) the nitrogen of fraction A2, and (*c*) the uncharacterized nitrogen of fraction B (Table 3) are designated "Unassigned N" in Table 1.

RESULTS

The saponification releases as free base most of the

TABLE 3. NITROGENOUS COMPONENTS OF THE CHLOROFORM PHASE (FRACTION B) AFTER SAPONIFICATION

Tissue	Total P	Total N	Uncharacterized N	Choline N	Ammonia N	Ethanolamine N	Serine N	Other Primary Amine N	Sphingosine N
Liver	1.18	10.0	1.63	1.79	0.69	0.87	0.0	0.98	4.06
Spleen	17.5	25.5	0.0	5.10	0.97	6.83	0.33	0.81	13.1
Pancreas	9.1	18.9	3.57	2.33	0.42	5.03	0.0	0.0	7.87
Lung	18.4	30.1	1.53	6.77	1.11	7.40	0.0	0.0	13.3
Lung	14.3	28.8	5.95	5.13	0.99	6.04	1.57	0.0	9.12
Kidney	20.1	28.6	0.18	6.51	1.01	10.0	0.0	0.0	10.8
Intestine	21.1	32.2	1.45	7.11	2.05	6.35	1.86	0.91	12.5
Heart	13.9	19.6	0.0	3.64	0.64	6.98	0.45	0.0	7.92

All values are given in per cent of total lipid N or P in the original extract.

nonsphingosine nitrogen from the lipids of these tissues, although the subsequent acidification with HCl is probably responsible for the hydrolysis of plasmalogen. Fractions A4, A5, A6, and A7 comprised 60% to 80% of the total lipid nitrogen, with only traces of phosphorus present. The remaining nitrogen is contained in sphingolipids and other alkali-resistant lipids in fraction B, and in acidic substances (including small amounts of phosphate ester) in fraction A3. The composition of the bases released by saponification is revealed by analysis of the five Dowex eluates, fractions A3 to A7 inclusive. A typical analysis for lipids of the intestine is shown in detail in Table 2. It is apparent that fractions A3, A4, and A5 contain, among them, all of the serine and other amino acids, nearly all of the substituted amines, and a part of the ammonia. Fraction A6 contains only ethanolamine and ammonia, and fraction A7 contains only choline and traces of ethanolamine. Hence, the minor components are entirely segregated into the first three Dowex fractions, which contain only 17% to 30% of the original total nitrogen. This circumstance allows for more accurate measurement of these substances, particularly of the substituted amines, which must be determined by difference. For this reason, the values in Table 1 for substituted amines in fractions A3, A4, and A5 are more valid than those which include fractions A6 and A7. Dowex 50 was used to separate ethanolamine from serine in lipid hydrolyzates by Dittmer *et al.* (21).

The amount of each base in the lipid is given as the sum of all the fractions (Table 1). Recovery of the total original lipid nitrogen is within 0% to 3% in all cases. All but a few per cent of this can be definitely assigned; only the "Unassigned N" remains uncertain.

The ammonia content of the aqueous phase after saponification is surprisingly high ("Saponification Ammonia," Table 1). This has been found consistently in lipid hydrolyzates by others, and has been attributed to decomposition of other bases, including ethanolamine and serine (21). The recovery of ethanolamine from acid hydrolyzates of several tissues may be unsatisfactory (2), but it is doubtful if significant amounts of ammonia arise from these two amines by the hydrolysis methods used in this study. Both ethanolamine and serine appear completely stable to the alkaline hydrolysis. The large amounts of ammonia, in some instances equivalent to over 20% of the ethanolamine found, must arise from other sources. Some may be derived from amide nitrogen in the peptide present, and some may be bound as an artifact of extraction. Ammonia is produced in additional amounts in the more drastic hydrolyses of fractions A3 and A5 (Table 2) and fraction B (Table 3). This is summarized in Table 1 as the difference between "Total Ammonia" and "Saponification Ammonia."

Total lipid hexosamine is also shown in Table 1. This appears largely in fractions A3 and A5. Since the total amount of this substance is small and scattered, not all of the fractions were analyzed. For uniformity, the values given for ethanolamine and serine, in fraction A5, are therefore not corrected for the small amounts of hexosamine present. In fraction A3 the hexosamine is presumably acylated and would be a component of the "Substituted Amines."

The values obtained for total choline are comparable to those published previously from this laboratory (22) and elsewhere. The choline found in lipids of fraction B (Table 3) might be used as a determination of sphingomyelin, since the saponification conditions are similar to those of Schmidt *et al.* (23), and since the plasmalogens are hydrolyzed upon acidification. The sphingomyelin values so obtained are lower than those reported by Schmidt *et al.* (23), who measured phosphorus after selective hydrolysis, and are much lower than those of Marinetti *et al.* (24), who used quantitative paper chromatography. Species and individual variations probably do not account for this magnitude of difference. Our analyses for sphingomyelin depend upon complete release of choline from sphingomyelin and may be low. On the other hand, a total phosphorus analysis might include other unknown alkali-stable phosphatides and give high values. The paper chromatographic values probably include other phosphatides.

The values for sphingosine are substantially lower, and those for ethanolamine correspondingly higher, than we have reported previously for dog tissues (2, 9, 19, 22). The two-stage hydrolysis gives more complete hydrolysis of an ethanolamine-containing lipid. The nature of this lipid is now under investigation. It is apparently not the glyceryl ether ethanolamine phosphatide isolated from egg yolk by Carter *et al.* (8). In liver, some of this ethanolamine is present in the phosphorus-free glycolipid fraction.[1]

The values for lipid serine are lower than we reported previously (2, 9), because of removal of interference from peptides and amino acids by the use of periodate (25). It is apparent that serine phosphatide is a minor component of these dog tissues, and especially of liver, heart, and pancreas. The low values for serine are in agreement with those of Collins and Wheeldon (26), based on hydrolysis of dinitrophenyl phospholipids and separation of the dinitrophenylamines. They are generally lower than those obtained by Nojima and

[1] J. M. McKibbin. To be published.

Utsugi (27) using dinitrophenyl derivatives prepared from lipid hydrolyzates, and by Marinetti *et al.* (24) using quantitative paper chromatography. Again, species and individual differences may account for a part of the variation, but much probably arises from differences in the methods of analysis. Moreover, the values for serine found in this study are maximal, since threonine and hexosamine would be determined as serine, and some of the serine may arise from peptide.

The amount of primary amine other than ethanolamine and serine is designated "Other Primary Amine" (Table 1), and is generally comparable to serine. This fraction is considered to be largely amino acid because it accompanies serine in the Dowex fractions A3, A4, and A5, and reacts with ninhydrin on the paper chromatograms at room temperature. None of the individual amino acids have been determined quantitatively. A number of different amino acids are present in the fractions. The basic amino acids are easily distinguished in these systems by their low R_f values, and have not been found in significant amounts in any of the chromatograms. The valine-leucine-isoleucine group is distinguished by the high R_f values and the characteristic appearance of the ninhydrin spots. This group is prominent in fractions A4 and A5 from all of the tissues. Although the total amount of free primary amine was increased by hydrolysis of fractions A3 and A5, appreciable amounts were free in the aqueous phase following saponification alone. Whether this amino acid and peptide represents a failure in purification, an artifact of extraction, or true lipid-bound amino acid in some stage of metabolism (28, 29) is unknown.

The quantity of "Substituted Amine" is subject to the error of analyses made by difference. The values from fractions A3, A4, and A5 are more reproducible than those from fractions A6 and A7. Material from fraction A3 has been prepared in quantity from dog and horse liver, and attempts have been made to identify the nitrogen-containing components. Small amounts of acylated hexosamine are present, but no sialic acid, as determined by the method of Svennerholm (30). The latter is absent in this same fraction from most of the other tissues, although a trace (0.2% of total lipid nitrogen) is present in lung. O-phosphoethanolamine and O-phosphoserine (21) are not included in this fraction, since they are determined as primary amine with the naphthoquinone sulfonate reagent. Small amounts of material were precipitated with sodium tetraphenylboron, mercuric chloride, picric acid, and reinecke acid. However, no major fraction was obtained in identifiable form.

In fractions A6 and A7, the values for substituted amine may represent artifact of analysis or unidentified lipid base, or both. Since much depends on the specificity of the reineckate determination of choline, this was re-examined in all of the fraction A7. Paper chromatograms showed single spots corresponding to choline in the three solvent systems. In addition, reineckates were prepared from $Ba(OH)_2$ hydrolyzates of whole lipid extracts of all of the tissues reported in this study. The basic material was regenerated from these salts by the method of Kapfhammer and Bischoff (31) and chromatographed on paper. Similarly, only choline spots were found from these salts using five solvent systems, and using Dragendorff's reagent and molybdic acid as developers.[2] It is probable that no quaternary amine other than choline is present, and reineckate choline nitrogen may be identified with Kjeldahl N. Inasmuch as monomethylethanolamine and dimethylethanolamine have been found in hydrolyzates of *Neurospora* (32) and rat liver lipids (33), these might account for a part of the substituted amine fraction. Collins (34) reported unidentified substituted amine in the lipids of sheep brain.

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REFERENCES

1. Engel, R. W., W. D. Salmon and C. J. Ackerman. In *Methods of Biochemical Analysis*, edited by David Glick, New York, Interscience Publishers, Inc., 1954, vol. 1, p. 265.
2. McKibbin, J. M. In *Methods of Biochemical Analysis*, edited by David Glick, New York, Interscience Publishers, Inc., 1959, vol. 7, p. 111.
3. Carter, H. E., D. S. Galanos and Y. Fujino. *Can. J. Biochem. and Physiol.* **34**: 320, 1956.
4. Folch, J., and D. D. Van Slyke. *J. Biol. Chem.* **129**: 539, 1939.
5. Wren, J. J. *Nature* **185**: 295, 1960.
6. Folch, J., and M. Lees. *J. Biol. Chem.* **191**: 807, 1951.
7. Huggins, C. G., and D. V. Cohn. *J. Biol. Chem.* **234**: 257, 1959.
8. Carter, H. E., D. B. Smith and D. N. Jones. *J. Biol. Chem.* **232**: 681, 1958.
9. McKibbin, J. M. *Federation Proc.* **16**: 835, 1957.
10. McKibbin, J. M., and W. E. Taylor. *J. Biol. Chem.* **178**: 17, 1949.
11. Folch, J., M. Lees and G. H. Sloane-Stanley. *J. Biol. Chem.* **226**: 497, 1957.
12. Moore, S., and W. H. Stein. *J. Biol. Chem.* **192**: 663, 1951.
13. Elson, L. A., and W. T. J. Morgan. *Biochem. J.* **27**: 1824, 1933.
14. Blix, G. *Acta Chem. Scand.* **2**: 467, 1948.

[2] R. Freebern and J. M. McKibbin. Unpublished data.

15. Frisell, W. R., and C. G. Mackenzie. In *Methods of Biochemical Analysis*, edited by David Glick, New York, Interscience Publishers, Inc., 1958, vol. 6, p. 63.
16. Folin, O., and R. D. Bell. *J. Biol. Chem.* **29**: 329, 1917.
17. Frame, E. G., J. A. Russell and A. E. Wilhelmi. *J. Biol. Chem.* **149**: 255, 1943.
18. Jeanloz, R. W., and E. Forchielli. *J. Biol. Chem.* **188**: 361, 1951.
19. McKibbin, J. M., and W. E. Taylor. *J. Biol. Chem.* **178**: 29, 1949.
20. Celmer, W. D., and H. E. Carter. *Physiol. Revs.* **32**: 167, 1952.
21. Dittmer, J. C., J. L. Feminella and D. J. Hanahan. *J. Biol. Chem.* **233**: 862, 1958.
22. McKibbin, J. M., and W. E. Taylor. *J. Biol. Chem.* **185**: 357, 1950.
23. Schmidt, G., B. Ottenstein, W. A. Spencer, K. Keck, R. Blietz, J. Papas, D. Porter, M. L. Levin, and S. J. Thannhauser. *A.M.A. J. Diseases Children* **97**: 691, 1959.
24. Marinetti, G. V., J. Erbland and E. Stotz. *Biochim. et Biophys. Acta* **30**: 642, 1958.
25. Artom, C. *J. Biol. Chem.* **157**: 585, 1945.
26. Collins, F. D., and L. W. Wheeldon. *Biochem. J.* **70**: 46, 1958.
27. Nojima, S., and N. Utsugi. *J. Biochem.* (*Tokyo*) **44**: 565, 1957.
28. Haining, J. L., T. Fukui and B. Axelrod. *J. Am. Chem. Soc.* **81**: 1259, 1959.
29. Gaby, W. L., R. N. Naughten and C. Logan. *Arch. Biochem. Biophys.* **82**: 34, 1959.
30. Svennerholm, L. *Biochim. et Biophys. Acta* **24**: 604, 1957.
31. Kapfhammer, J., and C. Bischoff. *Z. physiol. Chem., Hoppe-Seyler's* **191**: 179, 1930.
32. Hall, M. O., and J. F. Nyc. *J. Am. Chem. Soc.* **81**: 2275, 1959.
33. Bremer, J., and D. M. Greenberg. *Biochim. et Biophys. Acta* **35**: 287, 1959.
34. Collins, F. D. *Biochem. J.* **72**: 532, 1959.

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Fatty acids of human brain cerebrosides*

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SUMMARY

Four regions of two human brains were analyzed for the individual cerebroside acids: cerebral cortex white and gray matter, cerebellum (mainly gray matter), and corpus callosum. The total lipids were extracted from each section and passed through a column of Florisil to remove cholesterol and phosphatides. The crude cerebrosides were next passed through a column of mixed ion exchange resins to remove cerebroside sulfate and then purified by elution from a silicic-acid column. The acids were cleaved from the cerebrosides and separated into four classes: normal saturated, hydroxy saturated, normal unsaturated, and hydroxy unsaturated. The individual acids were then analyzed by gas-liquid chromatography. The cerebroside content of each brain region differed somewhat, as did the relative contents of hydroxy acids. However, the distribution of the acids within each class was rather independent of brain location. The normal saturated acids contained stearic and lignoceric as the major acids, but fairly large amounts of the C_{22}, C_{23}, and C_{25} acids were also present. The hydroxy saturated acids were similar, but contained little hydroxystearic acid. The unsaturated acids of both classes contained the C_{24} acid as the major constituent, together with considerable amounts of the C_{25} and C_{26} acids.

As part of a study of glycolipids, we have analyzed different regions of human brain. Although it has been known for a long time that there is a higher cerebroside concentration in white matter than in gray matter, nothing is known of the content of the individual fatty acids in the cerebrosides of different portions of the brain. A procedure for such analysis had been developed for rat brain (1) and this has been modified for use with human brain. The main modification involved replacement of the saponification step by the use of silicic-acid chromatography.

EXPERIMENTAL

Samples of frozen, unfixed human brain weighing 7 to 50 g were extracted and the lipid extracts purified with Florisil and ion exchange resins as previously described (1). The moderately pure cerebrosides were then prepared for application to a column of silicic acid[1] by making an intimate mixture with a small portion of the adsorbent. This was done by dissolving the lipids in chloroform-methanol 2:1 (v/v), adding the silicic acid (1.2 g/100 mg lipids), and removing most of the solvent with a rotary vacuum evaporator. The remaining solvent was removed by drying in air overnight and then for several hours at high vacuum (3).

A slurry of additional silicic acid (30 g/100 mg cerebrosides) was prepared in Skellysolve B (mainly hexane). The slurry was swirled 30 minutes to remove trapped air and poured in portions, with tapping, into a long glass column (1.8 or 2.2 mm inside diameter). The column was fitted at the bottom with glass wool, sand, and a very fine needle valve. The preparation of the packing was speeded by applying about 5 psi air pressure to the top of the column. The length of the packed region ranged between 70 and 95 cm. The silicic acid was conditioned in the column after the method of Weiss (4) by passing through chloroform-methanol 2:1 (v/v), 5.5 ml/gram silicic acid, then chloroform, 4 ml/gram silicic acid. The solvents were pumped through by means of a precision plunger-type pump, after the method of Spackman *et al.* (5). The means of connecting are shown in Figure 1.

The lipid sample, which had been dried in silicic acid, was then added as a slurry in chloroform and pumping was begun with chloroform-methanol 92:8 (v/v) at a flow rate of about 25 ml per hour. Fractions of 15 ml

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[1] Bio-Rad Laboratories, Richmond, Calif. Prepared according to the method of Hirsch and Ahrens (2) and used without further treatment.

each were collected and evaporated to dryness. The fractions comprising the major peak were pooled and analyzed for galactose content by the phosphoric acid-anthrone method (6).

The cerebrosides thus obtained (141 to 242 mg) were split by refluxing under a very slow stream of nitrogen with 10 ml of concentrated HCl and 50 ml of methanol. After 4 hours of heating, 50 ml of water was added and the mixture of fatty acids and esters was extracted with five 40-ml portions of ether. The ether extract was washed with water, evaporated to dryness under vacuum with the aid of toluene (to prevent splashing), and the residue esterified with dimethoxypropane, HCl, and methanol (7).

Following removal of the solvents under vacuum, the esters were separated into normal and hydroxy esters by chromatography with Florisil. Each fraction was then further separated into saturated and unsaturated groups with the aid of mercuric acetate (1). The mercuric acetate reaction was modified by allowing the reaction mixture to sit overnight at room temperature instead of heating. The hydroxy esters were converted to methyl ethers and all four fractions were analyzed by gas-liquid chromatography (1).

DISCUSSION OF THE METHODS

Isolation of the Purified Cerebrosides. Our previous work with rat brain cerebrosides had disclosed the presence of an impurity characterized by a carbonyl group, as shown by infrared spectra. The impurity could be removed by treatment with alkali and then ion exchange resins. However, this method failed in the case of human cerebrosides and we turned to silicic acid, which had recently become available in a much improved form. The silicic acid satisfactorily removed the carbonyl impurity and gave cerebrosides of reasonably good galactose and fatty acid content (Table 1). The sphingosine values were high, particularly in the second group of analyses (see below).

The silicic-acid column yielded some minor fractions, as well as a small amount of lipid trailing from the cerebroside peak. The trailing portion, which was discarded, contained only about 1% of the cerebroside hexose.

It was found that bubbles appeared in the silicic-acid column unless several pounds of pressure were maintained throughout the packing. This pressure was maintained by constricting the outlet with the needle valve at the bottom. In our earlier work we used a piece of Neoprene tubing and a screw clamp to adjust the resistance, but the swelling action of the solvent made it necessary to adjust the clamp frequently. The needle valve also needs one or two adjustments, but this is very easily done. It should be pointed out that this type of column restrictor withstands pressure much better and gives flow adjustments much more easily than the Teflon® stopcocks we have tried, and is in effect cheaper than these stopcocks because it can be removed readily and placed on the outlet of any other column fitted with quarter-inch glass capillary tubing.

The other metering valve (Fig. 1) was included in the

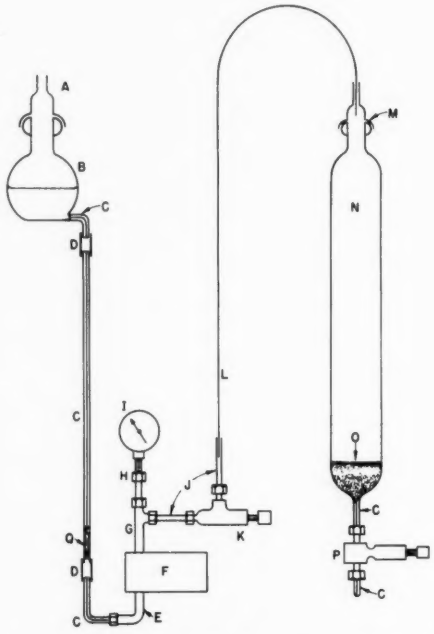

FIG. 1. Pumping system for lipid chromatography. *A*. Connection to compressed air, for priming the pump. *B*. Reservoir flask for solvent. Capillary tube, *C*, is offset to allow placing magnetic stirrer under flask when gradient elution is desired. *C*. Capillary glass tubing, nominally 6 to 7 mm diameter, chosen to fit through 1/4" Teflon Swagelok ferrules. *D*. Neoprene tubing, previously extracted with chloroform-ethanol in a Soxhlet extractor. *E*. Swagelok elbow, stainless steel, #400-2-2-316, with Teflon ferrules, #403-1 and 404-1 (Crawford Fitting Co., Cleveland 10, Ohio). *F*. Chromatographic miniPump, 1/8" plunger, 29 rpm, maximum capacity 124 ml/hour (Milton Roy Co., Philadelphia 18, Pa.). *G*. Swagelok tee, #200-3TMT-316. *H*. Swagelok adapter, #201-A-2F-316. *I*. Small pressure gage, 1/8" pipe connection. *J*. Short piece of 1/8" outside diameter stainless steel tubing. *K*. Metering valve, Nupro SS-2MA (Nuclear Products Co., Cleveland 10, Ohio). *L*. Stainless steel hypodermic needle tubing, 20 gauge. One end is soldered into the 1/8" steel tubing; the other end is cemented into a piece of capillary glass tubing with epoxy cement. The capillary tubing is previously sealed to an 18/7 socket. *M*. Neoprene sheet rubber gasket, 1/64" thick, made with two cork borers. The 18/7 joint is held together with two or three spring clamps. *N*. Chromatographic column, made of standard or heavy wall tubing. *O*. Glass wool and sand; base for packing. *P*. Metering valve, Nupro SS-4M, with 1/4" Teflon Swagelok ferrules. *Q*. Glass wool filter, to keep dirt out of pump.

pumping system to allow raising the resistance further, thereby increasing the accuracy of the pump (5). We adjusted this valve to keep the pump pressure at about 20 psi.

There is an appreciable volume of liquid in the pressure gauge; this tends to diffuse into the main stream of solvent rather slowly when a change is made to a solvent of higher density. For this reason we evacuated the pumping line for 15 minutes before changing from chloroform-methanol to chloroform.

The sample of cerebrosides was added to the silicic-acid column as a dried mixture in a little silicic acid because the lipid was too insoluble to be dislodged from the glassware by chloroform. Addition as a solution in chloroform-methanol (4) and elution with chloroform (of increasing methanol content) gives two cerebroside peaks instead of one. It is evident that this technique causes part of the cerebrosides to move with the initial portion of chloroform-methanol, and the remainder of the cerebrosides remain adsorbed until the methanol content of the eluting liquid is high enough to move them as an equilibrating sample. The finding by Weiss (4) that lignoceric acid could be isolated from the first peak and cerebronic acid from the second suggests that there is a preferential removal of the less polar cerebrosides in the solvent used for addition. This explanation was confirmed[2] by analyzing two such cerebroside fractions with the Florisil method. Both fractions contained normal acids, the content being somewhat lower in the second fraction.

TABLE 1. ANALYTICAL DATA FOR PURIFIED CEREBROSIDES

	1	2	3
	Galactose	Fatty Acids*	Sphingosine
	per cent	*per cent*	*per cent*
Cerebral cortex, white	20.9†	46.6	
	21.1	44.5	40.7
Cerebral cortex, gray	21.6	46.1	39.6
	21.4	45.2	41.7
Cerebellum	21.4	46.2	38.0
	21.5	44.9	44.4
Corpus callosum	23.6	48.5	39.8
	21.6	44.2	43.1
Theoretical values‡	21.6	46.5	35.9

* As methyl esters.

† In paired figures, top figure = brain from 70-year-old man; bottom figure = brain from 44-year-old man.

‡ Calculated from typical fatty acid analyses, assuming cerebrosides are monohydrate; MW = 835.

Splitting of the Cerebrosides. Ethanol-aqueous HCl has been used by Skipski *et al.* (8) for splitting off the acids of cerebrosides, and such a system is far more convenient than dry HCl mixtures. However, the formation of ethyl esters would make gas-liquid chromatography more difficult, so we substituted methanol for ethanol and raised the reflux period to 4 hours. Several trials had indicated this period was sufficient, but the low yields of esters from our second brain (Table 1, column 2) suggest that this period is too short. Sweeley and Moscatelli (9) have used 5 to 6 hours with methanol-HCl.

The crude esters obtained by ether extraction of the hydrolyzates weigh more than expected from theory and evidently contain some sphingosine, extracted possibly as the fatty acid salt. When the esters are separated with Florisil, the sphingosine is held back on the column and can be eluted with acetic acid-chloroform-methanol 3:4:4 (v/v). In the case of our second brain, the slightly low yields of esters were accompanied by a high yield of this sphingosine fraction. It seems likely that this fraction included ceramides. Although it is possible that incomplete hydrolysis may cause preferential splitting of one class of cerebrosides, the data for the fatty acid analyses do not indicate that this is an important effect.

The values in Table 1 for sphingosine were obtained by combining the weights of the two long-chain base fractions obtained by (*a*) taking the residual water-HCl-methanol solution after removal of esters, making it alkaline (Tropaeolin O indicator), and extracting with ether; and (*b*) eluting the Florisil column with acetic acid-chloroform-methanol and washing the chloroform with water, alkali, and water. The pooled sphingosine fractions were analyzed by Dr. Charles C. Sweeley, of the University of Pittsburgh, by gas-liquid chromatography (9).

It should be noted that the isolation method described here could be used equally well with much smaller amounts of brain, since no crystallization steps are involved. The large amounts were used mainly to help in validating the individual steps.

DISCUSSION OF THE DATA

The data obtained from the steps prior to gas chromatography are shown in Table 2.

Total Lipids and Cerebrosides. Columns 1 and 2, in

[2] Y. Kishimoto and N. S. Radin. Unpublished work.

TABLE 2. ANALYTICAL DATA FOR LIPIDS AND FATTY ACID CLASSES*

	1	2	3	4	5	6	7
	Total Lipids	Total Cerebrosides	Normal Acids	Hydroxy Acids	Hydroxy/ Normal	Saturated Normal/ Total Normal	Saturated Hydroxy/ Total Hydroxy
	per cent of wet brain		*per cent of cerebrosides*				
Cerebral cortex, white	21.2	3.36	15.6	31.0	1.99	0.58	0.57
	18.9	2.99	13.8	30.7	2.23	0.52	0.71
Cerebral cortex, gray	8.4	0.49	12.7	33.4	2.63	0.79	0.59
	8.5	0.47	5.4	39.8	7.37	0.70	0.71
Cerebellum	9.0		7.9	38.3	4.85	0.52	0.56
	8.6	0.67	12.3	32.6	2.65	0.47	0.69
Corpus callosum	21.4	3.52	20.7	27.8	1.34	0.51	0.64
	17.7	2.66	15.4	28.8	1.87	0.53	0.76

* See footnotes to Table 1 for explanatory notes.

agreement with the work of many others, show that white matter contains much more lipid and cerebrosides than gray matter. The difference is greater in the case of cerebrosides. Cerebellum resembles gray matter in this respect. The two brains differ in that the lipid and cerebroside concentrations are greater in the older brain, especially in the corpus callosum. Our figures for cerebroside concentrations are about one-third lower than those found by other workers (10, 11, 12), due mainly to the inclusion of cerebroside sulfate in the earlier analyses.

Hydroxy Versus Normal Acids. The values found for these two classes of acids are shown in columns 3 and 4, and the ratios of the two classes in column 5. As in rat brain cerebrosides (13), the hydroxy acids predominate. Cerebellum again resembles gray cortex, both showing somewhat greater predominance of hydroxy acids. Large individual differences are apparent in column 5, but we cannot state now whether these can be correlated with age.

Saturated Versus Unsaturated Acids. To conserve space, we present only the ratios of the concentrations. In column 6 we see that the degree of unsaturation in the normal acids is quite similar in both brains in all brain regions except gray cortex, where the saturated acids predominate. The degree of unsaturation is higher than that found in rat cerebrosides (13).

In the case of the hydroxy acids (column 7), there is marked similarity in all brain regions of any one brain. However, the younger brain consistently shows less unsaturation.

Gas-Liquid Chromatographic Data. Figures 2 to 5

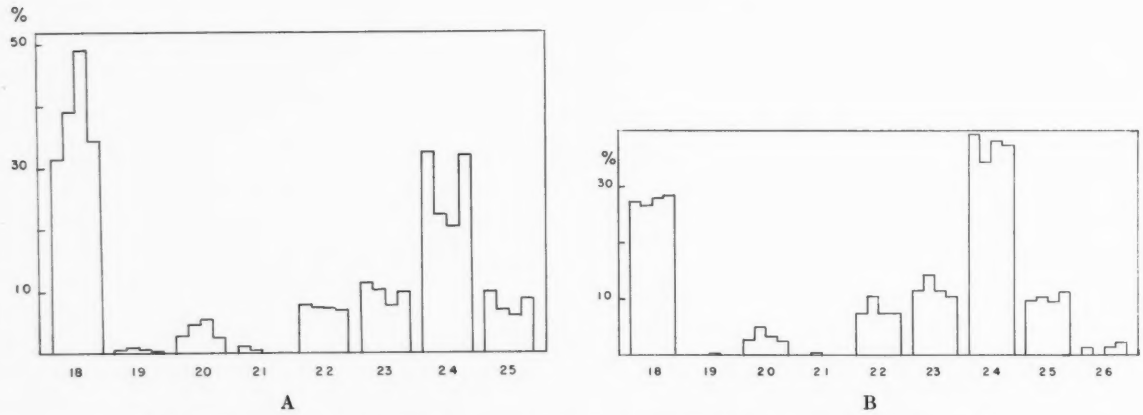

FIG. 2. Distribution of each normal saturated acid in brain cerebrosides, calculated as percentage of the total normal saturated esters. Each bar consists of four sections, corresponding (left to right) to cortex white, cortex gray, cerebellum, and corpus callosum. Figure 2*A* shows the results for the older brain; Figure 2*B*, for the younger brain. The trace of C_{19} acid in Fig. 2*B* is in the cerebellum and the C_{21} acid is in the cortex gray.

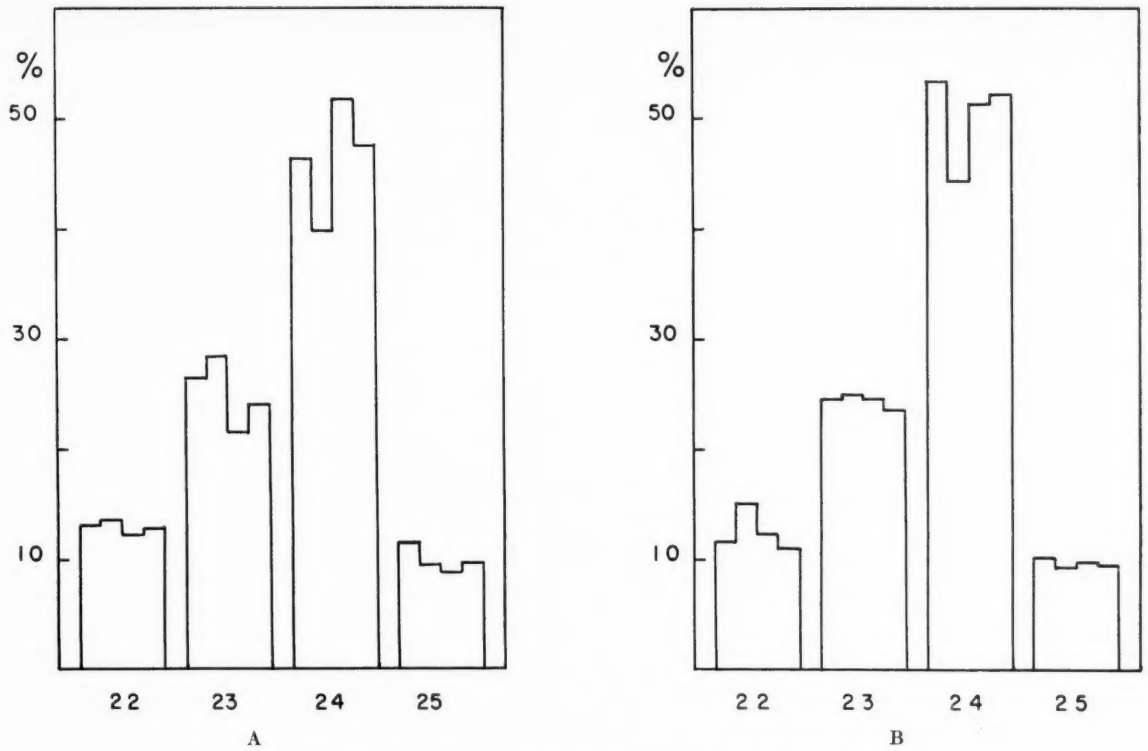

FIG. 3. Distribution of each hydroxy saturated acid. See Figure 2 for further description.

show the results of gas-liquid chromatographic analysis of each fraction. Excluded from the graphs are the longer and shorter acids that were found. Most of these occur in concentrations less than 1%, with a few over 2%. A slight distortion in the percentages arises from the use of esters and ester ethers, which have molecular weights differing from the acids originally present in the cerebrosides.

The normal saturated acids contain stearic and lignoceric acids as their major components, but appreciable amounts of the C_{22}, C_{23}, and C_{25} acids are also present. As in rat cerebrosides (1), the content of odd-numbered acids is unusually high. This high content explains much of the variability in analytical data reported for the C_{24} acids isolated from brain by conventional methods.

The hydroxy saturated acids also show a high content of C_{22}, C_{23}, and C_{25} acids. It has been found in rat brains that the concentrations of the odd-numbered acids increased with age (13), and the finding that the C_{23} hydroxy acid is the second major acid is consistent with the rat data. In contrast to the normal saturated acids, there is only a trace of the C_{18} acid, except in gray cortex. In this region there is a lower value for cerebronic acid that is accompanied by relatively high values (1.1% to 2.3%) for the C_{20}, C_{18}, C_{16}, and C_{14} hydroxy acids. This characteristic of gray cortex was found in both brains.

The unsaturated acids of both classes are notable for their high content of C_{25} and C_{26} acids, especially in comparison with the shorter acids. The C_{24} acids, however, play a much larger role in the unsaturated acids, as noted in our work with rat acids. Klenk and Leupold (14) have isolated the C_{26} normal unsaturated acid.

Trace amounts of unknown acids were observed in the two classes of hydroxy acids, mainly in the C_{19} region.

Particularly interesting is a comparison of the different brain regions with respect to the distribution pattern of the individual fatty acids in each class. This may be done by examining the profile of each bar in the bar graphs. Taking into consideration the variability intrinsic in the analytical method, one can see a marked similarity between the various brain regions. The similarity is even more marked when one reviews the large differences in cerebroside concentrations and ratios of hydroxyl to normal acids (columns 2 and 5, Table 2).

Only one serious exception to this similarity appears,

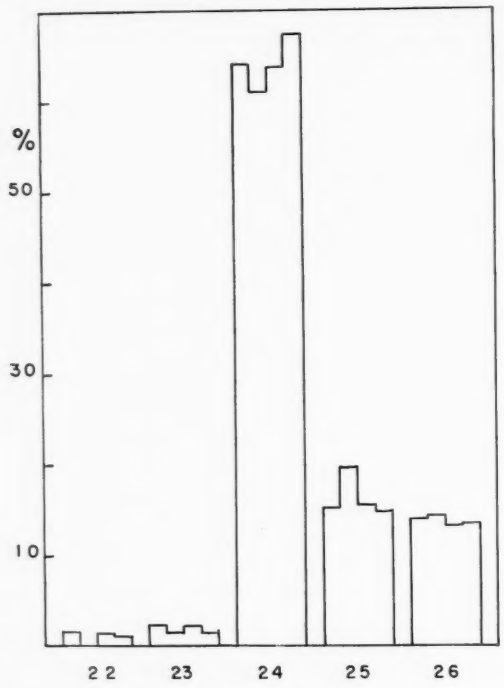

FIG. 4. Distribution of each normal unsaturated acid (younger brain only). See Figure 2 for further description.

in Figure 2*B*, where the stearic and lignoceric acid proportions differ somewhat in the gray cortex and cerebellum. There appears to be a small but consistent difference in all the cerebroside acids of gray cortex, where there is a little less of the C_{24} acids and more of the shorter acids. The effect is most noticeable, as mentioned before, in the hydroxy saturated acid group. Baker (15) has recently reported finding that the shorter acids of the nervous system (ester-linked) become more and more predominant with increasing distance from the center of the brain, a finding consistent with the above. Similarly consistent is the finding[3] that hydroxystearic acid is an important component of the spinal cord cerebrosides (8).

Although much more work is needed to clarify the significance of our data, we would like to offer a tentative hypothesis to explain the similarity in acid distribution between brain sections. If two or more types of cells synthesize the cerebroside acids, one would expect that each type would yield a different distribution pattern, that the two types of cells would occur in ratios differing with their location in brain, and that the distribution pattern would therefore differ from region to region. That the distribution of cell types does vary greatly with brain location is well known. It would appear, then, that only one cell type synthesizes the cerebroside acids (and presumably the cerebrosides too). The concept of a single cell type as the source of myelin has been proposed by Korey (16). This hypothesis also serves to explain the similarity in degree of saturation in the normal and hydroxy acids of the four brain areas (gray cortex again being the exception). However, one must then assume that the relative production of hydroxy acids is influenced by the location of the cells.

[3] A. K. Hajra and N. S. Radin. Unpublished work.

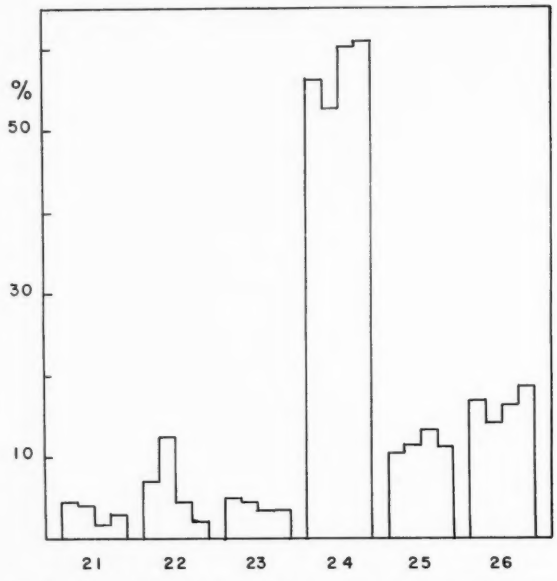

FIG. 5. Distribution of each hydroxy unsaturated acid (younger brain only). See Figure 2 for further description.

Analysis of the four sphingosine samples from one of the brains, kindly carried out by Dr. Sweeley, showed that 95% or more of the bases was sphingosine, about 2% was dihydrosphingosine, and a trace consisted of an unknown long-chain base. Here too the four brain areas were very similar and our postulate of a single cell type may be extended to cover the synthesis of the long-chain bases.

The authors wish to thank Dr. Harold Koenig, Veterans Administration Research Hospital, Chicago, for his kind aid in obtaining the brain sections.

REFERENCES

1. Kishimoto, Y., and N. S. Radin. *J. Lipid Research* **1**: 72, 1959.
2. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.

3. Radin, N. S. *Anal. Chem.* **28:** 542, 1956.
4. Weiss, B. *J. Biol. Chem.* **223:** 523, 1956.
5. Spackman, D. H., W. H. Stein and S. Moore. *Anal. Chem.* **30:** 1190, 1958.
6. Radin, N. S., F. B. Lavin and J. R. Brown. *J. Biol. Chem.* **217:** 789, 1955.
7. Radin, N. S., A. K. Hajra and Y. Akahori. *J. Lipid Research* **1:** 250, 1960.
8. Skipski, V. P., S. M. Arfin and M. M. Rapport. *Arch. Biochem. Biophys.* **82:** 487, 1959.
9. Sweeley, C. C., and E. A. Moscatelli. *J. Lipid Research* **1:** 40, 1959.
10. Cumings, J. N., H. Goodwin, E. M. Woodward, and G. Curzon. *J. Neurochem.* **2:** 289, 1958.
11. Brante, G. *Acta Physiol. Scand.* **18:** Supplementum 63, Table IX, 1949.
12. Johnson, A. C., A. R. McNabb and R. J. Rossiter. *Biochem. J.* **43:** 573, 1948.
13. Kishimoto, Y., and N. S. Radin. *J. Lipid Research* **1:** 79, 1959.
14. Klenk, E., and F. Leupold. *Z. physiol. Chem., Hoppe-Seyler's* **281:** 208, 1944.
15. Baker, R. W. R. *Biochem. J.* **78:** 29P, 1961.
16. Korey, S. R. *A.M.A. Arch. Neurol.* **2:** 140, 1960.

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Inhibition of cholesterol biogenesis by arsenite: preparation of labeled lanosterol*†

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SUMMARY

When mevalonic acid-2-C^{14} was incubated with rat liver homogenate with the usual cofactors plus 10^{-3} M arsenite, the formation of labeled cholesterol was inhibited and labeled lanosterol accumulated. This inhibitory effect of arsenite provides a convenient method of preparing labeled lanosterol in high yield (>10%) from commercially available C^{14}-labeled mevalonic acid.

It is well established that the biogenesis of cholesterol follows the following reaction sequence as reviewed by Popják and Cornforth (1):

MVA →→ squalene → lanosterol →→ cholesterol.

Although the steps between MVA and lanosterol have been largely elucidated in the past several years, much remains to be learned about the mechanism of conversion of lanosterol to cholesterol (1). One of the major problems in this area is the difficulty of obtaining labeled or unlabeled lanosterol. We wish to report the specific inhibition of the oxidation of lanosterol by arsenite, and a convenient method of preparing labeled lanosterol based on this effect.

MATERIALS AND METHODS

Racemic MVA-2-C^{14} of specific activity 1.1 μc/μmole was purchased from Tracerlab, Inc. Pure lanosterol was a kind gift of Professor K. Bloch. Rat liver homogenate was prepared and incubated with cofactors as described by Bucher and McGarrahan (2). Extraction and fractionation of nonsaponifiable material, and the determination of radioactivity in the sterols, in MVA and in CO_2 were as previously described (3, 4).

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† The following abbreviations are used: MVA, mevalonic acid; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide.

RESULTS AND DISCUSSION

When MVA-2-C^{14} was incubated under aerobic conditions with rat liver homogenate and the proper cofactors, substantial amounts of labeled CO_2 were formed. Addition of arsenite inhibited the formation of labeled CO_2 without affecting the yield in labeled nonsaponifiable material (Table 1). Chromatography of the nonsaponifiable material revealed that over 90% of the label was associated with the sterols, and that very little C^{14} was present in the squalene fraction. Since the conversion of MVA-2-C^{14} to lanosterol would not give rise to labeled CO_2, whereas the formation of cholesterol should be accompanied by the formation of radioactive CO_2, it appeared likely that the labeled product was lanosterol. This was confirmed by the following pieces of evidence: (*a*) When labeled

TABLE 1. EFFECT OF ARSENITE ON THE METABOLISM OF MVA-2-C^{14} IN RAT LIVER HOMOGENATE

Arsenite	CO_2		Sterols	
	Expt. A	Expt. B	Expt. A	Expt. B
	cpm	*cpm*	*cpm*	*cpm*
None	57,000	60,000	189,000	102,800
10^{-4} M	54,000		213,000	
10^{-3} M	5,400	6,000	160,000	90,900

10^6 cpm of racemic MVA-2-C^{14} (1 μmole) was incubated for 3 hours with 5 ml of homogenate. It can be seen that arsenite (10^{-3} M) strongly inhibited $C^{14}O_2$ production without affecting labeled sterol formation.

squalene, prepared by incubating MVA-2-C^{14} with rat liver homogenate under anaerobic conditions, was incubated with rat liver microsomes and soluble proteins, both labeled sterol and labeled CO_2 were formed. The addition of arsenite abolished the formation of CO_2 without affecting the yield of sterol (Table 2). (*b*) When the labeled sterol, obtained in the presence of arsenite, was recrystallized with cholesterol, the crystals obtained after two recrystallizations had no radioactivity. (*c*) When the labeled sterol was recrystallized with lanosterol, the radioactivity remained with the crystals after three recrystallizations.

TABLE 2. EFFECT OF ARSENITE ON THE CONVERSION OF SQUALENE TO CHOLESTEROL

Arsenite	CO_2		Sterols	
	Expt. A	Expt. B	Expt. A	Expt. B
	cpm	*cpm*	*cpm*	*cpm*
None	2,720	3,396	46,000	28,400
10^{-4} M	1,120	1,146	34,000	31,000
10^{-3} M	24	207	58,000	30,000

10^5 cpm of squalene was suspended with the aid of 0.5 mg of Tween 80 and incubated for 3 hours with 2 ml of dialyzed rat liver supernatant and microsomes. Addition of arsenite at 10^{-3} M inhibited only $C^{14}C_2$ formation. The squalene was prepared by the incubation of MVA-2-C^{14} with rat liver homogenate under anaerobic conditions. The supernatant and microsomes were obtained by centrifuging liver homogenate for 30 minutes at 8,000 × *g* to remove the mitochondria. The supernatant thus obtained was dialyzed for 3 hours at 2° against 200 volumes of 0.001 M phosphate buffer (pH 7.0).

Based on this inhibitory effector of arsenite, labeled lanosterol can be prepared conveniently from MVA-2-C^{14}. A typical experiment follows: Five milliliters of rat liver homogenate was preincubated with 10^{-3} M arsenite for 1 hour at 0°, and then incubated aerobically for 3 hours at 37° after the addition of 1 μmole of racemic MVA-2-C^{14}, 10 μmoles of DPN^+, and 20 μmoles of ATP. After saponification, extraction, and chromatography on active alumina, the yield of lanosterol was 0.2 μc, or 40% of the maximum theoretical yield.

REFERENCES

1. Popják, G., and J. W. Cornforth. *Adv. Enzymol.* **22:** 281, 1960.
2. Bucher, N. L. R., and K. McGarrahan. *J. Biol. Chem.* **222:** 1, 1956.
3. Tchen, T. T., and K. Bloch. *J. Biol. Chem.* **226:** 921, 1957.
4. Olson, J. A., Jr., M. Lindberg and K. Bloch. *J. Biol. Chem.* **226:** 941, 1957.

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Cholesterol and mevalonate metabolism: a time course study *in vivo**

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SUMMARY

Within 30 minutes after injecting DL-mevalonate-2-C^{14} into young rats, the total body nonsaponifiable fraction contained 47.6% of the biologically active C^{14}-mevalonate. Time course data suggest that at this time utilization of tracer MVA was essentially complete. Urinary radioactivity accounted for slightly more than one-half of the dose. The metabolism of nonsaponifiable fractions of liver and gut may be related by two processes having similar but reciprocal half times. Kidney tissue was found to contain the greater part of the radioactivity previously associated with carcass nonsaponifiable fractions. A small but consistent amount of label was found in the saponifiable fractions.

The problem of cholesterol and fatty acid biosynthesis, transport, and turnover has been under investigation in this laboratory for some time (1 to 5), the larger part of these studies having been made with acetate-1-C^{14} as the labeling agent. The multiple pathways available for acetate metabolism tend to obscure the information desired on any single pathway, particularly if the time periods involved allow for recycling of the tracer.

It appears well established that mevalonic acid (MVA) is a unique and a more direct precursor of squalene and cholesterol in mammalian systems (6 to 15) than is acetate. While most of these studies have been made with homogenates, we have compared acetate and mevalonate as tracers in a liver slice system (1). In the homogenate and liver slice systems, using C^{14}-acetate and C^{14}-mevalonate as tracers, it is possible to obtain definitive information largely on biosynthetic pathways. We have shown that the *in vivo* and *in vitro* methods are not directly comparable for studies on cholesterol metabolism (16), and that both *in vivo* cholesterol transport and turnover may be important facets of cholesterol metabolism (2).

The present experiments were designed to utilize the more selective labeling of cholesterol by mevalonate in studies on cholesterol biosynthesis and turnover in the intact rat. This paper presents data on the early time course of the labeling of the nonsaponifiable fraction in normal rats injected with MVA-2-C^{14}. Included in the presentation are data on the amount of label appearing in CO_2, blood, and urine during the first 4 hours after tracer administration. A detailed analysis of the participation of the tissues of the carcass is also presented.

It is shown that the intact rat quickly utilizes tracer MVA-2-C^{14} for sterol synthesis. The studies reveal that the turnover rates of liver and gut nonsaponifiable fractions seem to be reciprocally related, the processes, moreover, having half times shorter than any of those described by others. Kidney is shown to convert a seemingly disproportionate amount of MVA-2-C^{14} to nonsaponifiable material.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Northwest Rodent Co., Pullman, Wash.) were maintained on Purina Chow for at least 5 days, and were then trained to feeding (5). Animals weighed 80 to 100 g at the time of use. Young animals were utilized because of the observation of Wright and Cleland (9) that liver homogenates from young rats favored incorporation of MVA into cholesterol. In our laboratory, work *in vivo* has indicated, however, that 200-g young adult rats show essentially the same magnitude and pattern of labeling as do the 80-g animals. Following a 1-hour feeding period and a 1-hour fast, animals were injected

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intraperitoneally with 0.42 μc contained in 0.46 μmole of DL MVA-2-C^{14} (Tracerlab, Inc.). This nutritional regimen was followed to minimize the variations in lipogenesis which are in part dependent upon fasting and feeding periods (4, 5, 17). In the present studies 30 animals were used.

Following the injection of label, the animals were maintained in metabolism cages for urine collection.[1] Respiratory CO_2 was collected for various periods on five animals, using the apparatus previously described (18), which allows for total CO_2 collection and also continuously monitors the $C^{14}O_2$ activity. After the specified time interval, the animals were killed by decapitation, at which time blood was collected. Initially, the animals were separated into the four tissue fractions: liver, gut, carcass,[2] and skin. In subsequent experiments the following tissues comprising the carcass fraction were separately studied: kidney, adrenal, epididymal fat pad, brain, lung, spleen, leg muscle, diaphragm, heart, and testes.

All tissues were saponified, and lipid fractions separated as described earlier using an aliquot technique (17). The amounts of fatty acid, the saponifiable fraction, were determined gravimetrically. The nonsaponifiable material was determined colorimetrically[3] by the method of Zlatkis *et al.* (19), using cholesterol as the standard. In our hands these fractions gave essentially identical values when either the Zlatkis color or the Liebermann-Burchard color was used.

The lipid fractions were radioassayed as infiritely thin samples with a Nuclear-D47 Micromil gas flow counter. The radioactivity of the blood and urine samples was assayed as liquid samples (20). The radioassay values were converted to their infinitely thick $BaCO_3$ equivalents, using factors determined in this laboratory. The total activity of the blood was calculated, using 8% of the body weight of the rat as the total blood volume.

[1] It was found during the course of these studies that the urinary excretion of radioactivity is a good indication of a successful intraperitoneal injection. If the injection is made incorrectly, i.e., into the gut or into some other tissue mass, there is a delayed urinary excretion of activity. The occasional animals which excreted less than 40% of the labeled dose in the first hour were not used for the present series because nonsaponifiable fraction labeling in these animals directly paralleled the excreted dose up to a maximum of 23% incorporation.

[2] Residual tissue of the entire animal after removal of liver, gut, and skin. The carcass fraction also contains the blood collected at the time of decapitation.

[3] In this paper the word cholesterol is used to describe the material colorimetrically determined in nonsaponifiable fractions. In our hands the colorimetric determination and radioassay of other nonsaponifiable fractions of liver, gut, carcass, and skin fractions, one and two hours after giving mevalonate-2-C^{14} to intact animals, indicate that better than 90% of the C^{14} is in the form of digitonin precipitable, color-reacting sterol. Since our present interests are in cholesterol or related sterols, the use of digitonin was omitted.

RESULTS

In the upper portion of Table 1 are recorded, as cholesterol, the amounts of nonsaponifiable material found in the major tissues studied, and in the lower portion the values for the various tissues of the carcass fraction. Carcass tissue contains 55% of the animal's total cholesterol, while the gut, skin, and liver contains 23%, 16%, and 6%, respectively. It is of interest that the amount of cholesterol per gram of tissue is relatively constant in the four fractions cited above. Brain and adrenal tissue each have an appreciably higher cholesterol concentration than other tissues. The amounts of cholesterol in the various fractions are similar to those found by Chevallier (21).

TABLE 1. CHOLESTEROL CONTENT OF TISSUE AND DISTRIBUTION OF BODY CHOLESTEROL

Fraction	No. of Animals	Tissue Weight	Cholesterol		Total Body Cholesterol
		g	*mg/g*	*mg*	*per cent*
Liver	16	4.5	2.6* ± 0.6	11.7	6
Gut	16	21.4	2.0 ± 0.6	42.8	23
Skin	17	10.3	2.9 ± 0.9	29.9	16
Total carcass†	17	51.7	2.0 ± 0.3	103.4	55
Kidney	7	0.88	4.3* ± 0.4	3.8	2.0
Brain	4	1.70	13.1 ± 2.9	22.3	11.9
Adrenals	8	0.02	34.5 ± 11.0	0.7	0.4
Diaphragm	3	0.32	0.73‡	0.2	0.1
Leg muscle	3	1.00§	0.85	0.9	0.5
Testes	2	0.84	1.90	1.9	0.9
Epididymal fat pads	2	0.80	0.51	0.4	0.2
Lung	2	0.64	4.50	2.9	1.5
Spleen	2	0.31	3.81	1.2	0.6
Heart	3	0.34	1.33	0.5	0.2

* Mean value ± standard deviation.

† Residual tissue of the whole animal after removal of the liver, gut, and skin.

‡ Mean value.

§ One gram of muscle tissue was used. The total muscle and bone mass accounts for the remaining cholesterol listed above as being present in the carcass fraction.

Table 2 presents information on the total amounts of the administered dose found in various fractions through the 4-hour time period. The molecular species

TABLE 2. RECOVERY OF INJECTED MEVALONATE-2-C^{14} RADIOACTIVITY*

Time	No. of Animals	Fractions					Total Recovery
		CO_2†	Nonsaponifiable‡	Saponifiable‡	Urine§	Blood§	
15 min	3	0.46	15.9	2.5	16.0	7.0	41.9
30 min	3	1.44	22.7	3.0	23.3	5.0	55.4
1 hour	3	3.50	23.8	3.8	52.5	2.5	85.8
2 hours	8	6.40	21.4	2.3	59.6	2.4	92.3
3 hours	3	8.00	21.9	3.3	58.6	2.5	94.3
4 hours	5	9.00	18.0	3.6	57.0	2.7	90.4

* Per cent of the total dose of DL MVA-2-C^{14}.

† The CO_2 data were obtained from a total of 5 animals, the serial samples being so spaced in time as to yield a minimum of three values for each time interval.

‡ Sum of activities of all tissues, i.e., total animal activity.

§ Total radioactivity of the urine collected after injection, and of the total blood volume.

containing the radioactivity of the blood and urine were not characterized, but it is possible that the greater part of the activity in the urine is the biologically inactive isomer of MVA. The first observation of interest from Table 2 is that in 30 minutes nonsaponifiable fraction label reached 95% of the maximum which was found at the 1-hour postinjection time.

It appears that about one-fourth of the tracer dose (or about one-half of the biologically active isomer) is the maximum which can be incorporated into the nonsaponifiable fraction under the present conditions. Of the total dose, 3.8% is directed to the saponifiable fraction, and again in this fraction an early maximum is reached. Urinary excretion of C^{14} activity is 90% complete within the first hour, paralleled by a decrease in radioactivity of the blood to a low level at this time. At the 15- and 30-minute time intervals, total recovery of activity is only 41.9% and 55.4%, respectively. It is assumed that the activity unaccounted for in this total is as yet unused MVA per se. We have shown previously (1) that MVA remains in an aqueous phase, normally discarded in our fractionation procedure, and does not appear either in our saponifiable or nonsaponifiable fractions. By the end of the first hour, 86% of the injected radioactivity was recovered. The excretion of $C^{14}O_2$, as indicated in Table 1, is shown to increase during the 4 hours and differences between these figures, each normalized to periods of 1 hour, reveal that a maximum rate was obtained at 1 hour, the subsequent intervals showing a decreased rate of $C^{14}O_2$ excretion.

Table 3 presents data on the radioactivity found in the nonsaponifiable fractions of liver, gut, carcass, skin, and brain over a 24-hour period following injection. The radioactivity of liver, carcass, and skin fractions reached maximum values within a short time. The brain tissue of these intact animals is relatively inert as regards lipid synthesis, even in these young animals. There was a loss of nonsaponifiable fraction activity from the liver after the early maximum, and there was a concomitant increase in the labeling of the corresponding fraction of gut. If the information concerning liver

TABLE 3. TIME COURSE OF NONSAPONIFIABLE FRACTION LABELING FROM MVA-2-C^{14}

Time*	No. of Animals	Incorporation into Nonsaponifiable Lipid				
		Liver	Gut	Carcass	Skin	Brain
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
15 min	3	4.0†	1.1	10.1	0.4	0.04
		(3.3–4.7)	(0.8–1.7)	(8.9–11.2)	(0.3–0.4)	
30 min	3	4.9	1.3	16.0	0.7	0.03
		(4.6–5.2)	(1.1–2.9)	(15.2–16.7)	(0.5–0.8)	
1 hour	3	4.6	1.8	17.1	0.6	
		(4.1–5.7)	(1.5–2.0)	(16.3–17.9)	(0.6)	
2 hours	4	2.3	2.6	17.4	0.5	
		(1.8–2.5)	(2.4–2.7)	(16.7–18.5)	(0.5–0.6)	
3 hours	3	2.5	2.9	16.0	0.5	0.05
		(1.9–3.1)	(2.4–3.4)	(14.9–17.0)	(0.5)	
4 hours	3	1.8	3.0	13.0	0.5	0.06
		(1.2–2.4)	(2.9–3.1)	(9.5–16.5)	(0.5)	
24 hours	2	1.1	2.6	16.6	0.6	
		(0.9–1.5)	(2.4–2.8)	(16.5–16.6)		

* Time animal was sacrificed following injection.

† Mean (upper figure) and range of values (figures in parentheses).

and gut (Table 3) is plotted as in Figure 1, biphasic reciprocal curves appear to be present in these fractions. The liver fraction has a rapid component with a half life ($t^1/_2$) of 1.3 hours, and a slower component with a $t^1/_2$ of 5.0 hours. The gut fraction is resolvable into two components that have half lives of 1.5 and 8.0 hours.

Because of the cellular and metabolic heterogeneity of the carcass fraction, it was felt desirable to define more precisely the participation of the different tissues. Table 4 records the values obtained on the distribution

TABLE 4. DISTRIBUTION OF LABELED NONSAPONIFIABLE RADIOACTIVITY 2 HOURS POSTINJECTION

Fraction	No. of Animals	Total*	Fraction	No. of Animals	Total*
		per cent			*per cent*
Kidney	4	64.0† (46.0–77.0)	Lung	3	0.7 (0.5–1.0)
Carcass‡	4	10.6 (7.0–15.4)	Spleen	3	0.5 (0.3–0.8)
Gut	4	12.1 (11.4–12.6)	Diaphragm	3	0.2 (0.1–0.3)
Liver	4	10.7 (8.4–11.7)	Heart	2	0.2 (0.1–0.3)
Skin	4	2.5 (2.3–2.5)	Testes	3	0.2 (0.1–0.3)
Adrenal	2	0.2 (0.1–0.2)	Muscle§	4	0.2 (0.1–0.5)
Epididymal fat pads	3	0.2 (0.2–0.3)			

* Per cent of the animals' total nonsaponifiable radioactivity found in the indicated tissues.

† Mean (upper figure) and range of values (figures in parentheses).

‡ Residual muscle mass and bone structure after removal of the other tissues listed.

§ One gram of tissue.

of labeled nonsaponifiable material 2 hours after mevalonate injection. The results for the liver, gut, and skin fractions agree well with the previous data for animals 2-hour postinjection (Table 3). A most interesting finding is the relative amount of radioactivity found in the nonsaponifiable fraction of kidney tissue. The other fractions of the carcass contribute relatively little of the radioactivity of the nonsaponifiable fraction when compared to the kidney.

With the finding that at 2 hours the kidney contained the greater part of the radioactivity of the carcass fraction, it appeared desirable to define more precisely the role of the kidney in cholesterologenesis. To this end, a special time course study was made to follow

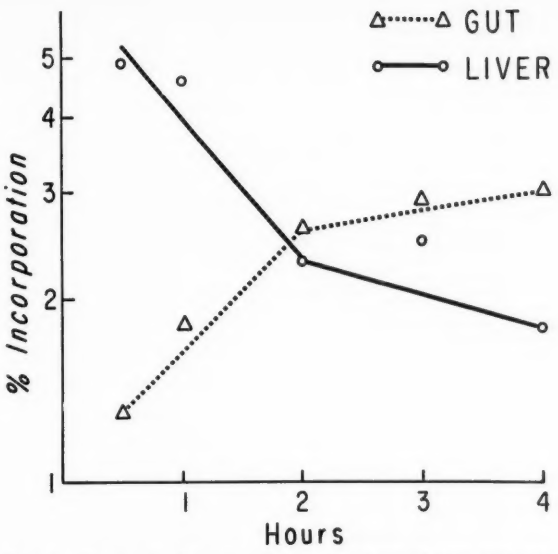

FIG. 1. Turnover of nonsaponifiable fractions of liver and gut. The logarithms of the per cent incorporation of MVA-2-C^{14} into nonsaponifiable fractions have been plotted with respect to time after injection. The straight line functions were obtained by the method of least squares. The half life of the fast component of liver is 1.3 hours, and of the slower component 5.0 hours. The more rapid component of gut tissue has a half life of 1.5 hours, and the slower component one of 8.0 hours.

the metabolism of the nonsaponifiable fraction in kidney tissue. From the data of Table 5 it is clear that at

TABLE 5. TIME COURSE DATA ON TOTAL CARCASS AND ON KIDNEY TISSUE

Time	No. of Animals	Incorporation of MVA-2-C^{14} into Carcass		Carcass Activity Found in Kidney	
		Nonsaponifiable Fraction	Saponifiable Fraction	Nonsaponifiable C^{14}	Saponifiable C^{14}
		per cent	*per cent*	*per cent*	*per cent*
15 min	2	12.5* (11.8–13.3)	0.9†	79.1	36.0
1 hour	2	17.9 (17.2–18.6)	1.5†	75.2	39.5
2 hours	4	13.9 (9.9–16.6)	0.7 (0.31–1.21)	65.0	29.2
3 hours	1	15.6 (15.6)	0.9†	71.0	27.3
4 hours	2	13.0 (12.8–13.2)	0.6 (0.5–0.6)	72.2	16.7

* Mean value (upper figure) and range of values (figures in parentheses).
† Single determinations.

the 15-minute time the kidney contains some 79% of the radioactivity generally attributed to the carcass nonsaponifiable fraction. This radioactivity remained high during the 4-hour period of study. Some 40% of the total carcass saponifiable fraction was also found in kidney tissue at the 1-hour time interval.

DISCUSSION

The distribution of cholesterol in four rat tissues of liver, gut, carcass, and skin has been followed and reported from this laboratory in previous publications (2 to 5). The present investigation has shown the total carcass to be a metabolically heterogeneous system. The adrenal gland is shown to contain the highest concentration of color-producing sterol. The brain contains by far the largest total quantity of sterol. Liver, gut, skin, kidney, testes, lung, spleen, and heart contain lesser amounts of cholesterol per gram of tissue, but of this last group, kidney contains the highest. In contrast to the organ structures, muscle contains only small amounts of cholesterol per gram of tissue.

The information presented in Table 2 shows that 30 minutes after the intraperitoneal injection of MVA-2-C^{14}, 22.7% of the C^{14} dose is found in the nonsaponifiable fraction, 3% in the saponifiable fraction, 23.3% in the urine, 5% in the blood, and 1.44% in the CO_2, giving a total recovery of 55.4% of the injected dose. In the next 30 minutes the animals seemingly utilized all the available MVA. In other words, labeling of the nonsaponifiable fraction from MVA would appear to be essentially complete after 30 minutes, so that any major change in the radioactivity of this fraction should be due to transport or metabolism. It is also likely that the major part of the label was now in cholesterol fractions because calculations made, as before (1), reveal that the $C^{14}O_2$ activity at 1 hour represents the conversion of 0.0081 μmole of L-mevalonate to CO_2. Considering the radioactivity of the nonsaponifiable fraction at this time to represent true cholesterol activity, one would expect the release of some 0.01 μmole of $C^{14}O_2$ by the demethylation of lanosterol to cholesterol. The similarity in magnitude of these observed and calculated figures is of interest.

It was originally thought that the radioactivity in the saponifiable fraction might consist of intermediates in cholesterol synthesis (22), i.e., farnesyl pyrophosphate, but the maintenance of this activity over the 4-hour period would tend to rule this out. If the activity was present in obligate precursors of cholesterol, there would probably have been diminished amounts of label remaining in the saponifiable fraction after 30 minutes. It can be concluded that this activity constitutes substances not on the direct cholesterol biosynthesis pathway, and perhaps similar to the acid described by Ogilvie and Langdon (23, 24). Elwood *et al.* (1) and Popják (22) have previously recorded activity of saponifiable components from MVA-2-C^{14} labeling.

The total radioactivity of the blood diminishes rapidly during the first 30 minutes, probably reflecting the combination of rapid tissue uptake, rapid synthetic reactions, and also urinary clearance during the same period. The activity in the blood during the first half hour probably results chiefly from both D- and L-MVA. The information reported in Table 3 on the amount of label in the various tissue nonsaponifiable fractions may represent the results of both synthesis and mobilization of lipid. The radioactivity present in the liver declines at a rapid rate during the first 2 hours, the liver losing approximately one-half of its radioactivity in this period. This loss appears to be caused by at least two processes, the more rapid one having a half life of about 1.3 hours, and the slower one a half life of 5.0 hours. In other time course studies (2), there were shown to be two components of decay, one with a half life of about 15 hours and a slower component of 8.9 days. The two rapid components described in the present study were, of course, not detectable in the previous long-term experiment. When the two studies are considered together, the metabolism of nonsaponifiable lipid in the liver may be described as the resultant of a minimum of four processes, having half times of 1.3, 5.0, 15, and 210 hours.

The gut is seen to increase in labeled nonsaponifiable radioactivity during the 4-hour interval. Figure 1 shows a biphasic-type curve for this response, the half life of the fast component being 1.5 hours and that of the slow component being 8.0 hours. Although it is not possible to equate arithmetically the radioactivity present in the gut and liver tissues, it would seem likely that at least a portion of the newly synthesized sterol was not in equilibrium with total liver sterol, and was, in fact, selectively secreted from the liver. The 5.8% of the tracer dose present in nonsaponifiable material in the gut at the 15-minute time interval would seem to indicate that appreciable cholesterol synthesis had occurred. As shown previously in this laboratory (25), the use of the intraperitoneal route of tracer acetate administration resulted in gut tissue containing more of the label than from the intravenous route of dosage. As yet, it is not clear if this results from a local utilization of tracer before systemic distribution is effected, or whether it is caused by selective distribution to certain tissues.

The skin appears to synthesize a small amount of nonsaponifiable material during the initial time when MVA is available to it. However, no further increase or decrease is seen in the activity during the time of observation. In the previous studies (2), nonsaponifiable radioactivity of skin increased in the first 4-day period. This slower response may not be detectable in the short time of the present experiments. The brain

was quite inert as regards synthesis from MVA, or in storage of newly formed nonsaponifiable material. The activity reported here for brain may actually represent residual blood activity.

It is clear that the kidney is the tissue of the carcass which is responsible for the maximum nonsaponifiable labeling from MVA in these rats, since 79% of the carcass nonsaponifiable activity was found in the kidney at the end of 15 minutes, and this activity remained essentially constant during the 4-hour period under investigation.

These findings on the kidney tissue should not be directly interpreted as representing an unduly great synthesis of nonsaponifiable material. The intact kidney may collect, and thus concentrate, the labeled MVA, and in this fashion increase the amount of C^{14} diverted to biosynthesis in this tissue. Similar findings in the saponifiable fraction, i.e., 36% of the activity in the total acidic material found in the kidney at the 15-minute time interval, would also indicate the increased availability of label to kidney tissue. Unfortunately, without knowledge of the specific activity of the precursor pools of the individual tissues, it is not possible to compare directly the rates of synthesis of various tissues. In other *in vitro* studies (unpublished data), active labeling of nonsaponifiable and saponifiable components from mevalonic acid in kidney tissue was confirmed.

From previous work in our laboratory (2) it can be calculated that 15.8%, 35.2%, 34.9%, and 14.1% of the labeled nonsaponifiable material was in the liver, gut, carcass, and skin, respectively, 2 hours following injection of acetate-1-C^{14}. The present work lists values of 11.2%, 13.2%, 77%, and 2.7% for similar fractions labeled by MVA-2-C^{14}. It becomes evident that the labeling pattern of body lipid is grossly different when MVA-2-C^{14} is the precursor than when acetate-1-C^{14} is used.

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REFERENCES

1. Elwood, J. C., A. Marcó and J. T. Van Bruggen. *J. Biol. Chem.* **235**: 573, 1960.
2. Wong, R. K. L., and J. T. Van Bruggen. *J. Biol. Chem.* **235**: 30, 1960.
3. Van Bruggen, J. T., T. T. Hutchens, C. K. Claycomb, W. J. Cathey, and E. S. West. *J. Biol. Chem.* **196**: 389, 1952.
4. Hutchens, T. T., J. T. Van Bruggen, R. M. Cockburn, and E. S. West. *J. Biol. Chem.* **208**: 115, 1954.
5. Cockburn, R. M., and J. T. Van Bruggen. *J. Biol. Chem.* **234**: 431, 1959.
6. Tavormina, P. A., M. H. Gibbs and J. W. Huff. *J. Am. Chem. Soc.* **78**: 4498, 1956.
7. Tavormina, P. A., and M. H. Gibbs. *J. Am. Chem. Soc.* **78**: 6210, 1956.
8. Dituri, F., J. L. Rabinowitz, R. P. Hullin, and S. Gurin. *J. Biol. Chem.* **229**: 825, 1957.
9. Wright, L. D., and M. Cleland. *Proc. Soc. Exptl. Biol. Med.* **96**: 219, 1957.
10. Isler, O., R. Ruegg, J. Wursch, K. F. Gey, and A. Pletscher. *Helv. Chem. Acta* **40**: 2369, 1957.
11. Cornforth, J. W., R. H. Cornforth, G. Popják, and I. Y. Gore. *Biochem. J.* **69**: 146, 1958.
12. Popják, G., L. Gosselin, I. Y. Gore, and R. G. Gould. *Biochem. J.* **69**: 238, 1958.
13. Amdur, B. H., H. Rilling and K. Bloch. *J. Am. Chem. Soc.* **79**: 2646, 1957.
14. Rilling, H., T. T. Tchen and K. Bloch. *Proc. Natl. Acad. Sci. U.S.* **44**: 167, 1958.
15. Bloch, K. In *Ciba Foundation Symposium on Biosynthesis of Terpenes and Sterols*, edited by G. E. W. Wolstenholme and M. O'Connor, London, J. & A. Churchill, Ltd., 1959, p. 6.
16. Emerson, R. J., W. C. Bernards and J. T. Van Bruggen. *J. Biol. Chem.* **234**: 435, 1959.
17. Emerson, R. J., and J. T. Van Bruggen. *Arch. Biochem. Biophys.* **77**: 467, 1958.
18. Van Bruggen, J. T., and T. T. Hutchens. U.S. Atomic Energy Commission Release AECU 3128.
19. Zlatkis, A., B. Zak and A. J. Boyle. *J. Lab. Clin. Med.* **41**: 486, 1953.
20. Marcó, A., J. C. Scott, J. C. Elwood, and J. T. Van Bruggen. *Anal. Chem.* **31**: 1746, 1959.
21. Chevallier, F. Ph.D. dissertation, University of Paris, 1956.
22. Popjak, G. In *Ciba Foundation Symposium on Biosynthesis of Terpenes and Sterols*, edited by G. E. W. Wolstenholme and M. O'Connor, London, J. & A. Churchill, Ltd., 1959, p. 148.
23. Ogilvie, J. W., Jr., and R. Langdon. *J. Am. Chem. Soc.* **81**: 754, 1959.
24. Ogilvie, J. W., Jr. *J. Am. Chem. Soc.* **81**: 756, 1959.
25. Williams, W. R., and J. T. Van Bruggen. *Am. J. Physiol.* **186**: 393, 1956.

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The effect of dietary fatty acids on coprostanol excretion by the rat*

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SUMMARY

By means of gas-liquid chromatographic analysis, coprostanol excretion has been studied in rats fed diets containing either no fat or varying amounts of linoleic acid, palmitic acid, or oleic acid. Coprostanol excretion was accelerated by linoleic acid, and depressed by oleic and palmitic acids. The acceleration of coprostanol formation by linoleic acid was demonstrated to occur in the large intestine. In these experiments cholesterol and coprostanol were the only neutral excretion products of cholesterol-4-C^{14}.

The mechanisms by which variations in the type and quantity of dietary fat alter cholesterol metabolism in both man (1, 2, 3) and experimental animals (4, 5) are unknown. Previous attempts to evaluate the influences of various unsaturated fats on cholesterol metabolism, both in our laboratory (6, 7) and by others (8, 9) have been complicated by two factors—the cumbersome methods required for the separation of various cholesterol excretory products and the fact that the natural sources of unsaturated fats contain a variety of plant sterols (10) that interfere with these determinations. The recent development of relatively simple gas-liquid chromatographic techniques for the separation of sterols (11) and the current availability of highly purified unsaturated fatty acids in quantities sufficient for dietary studies (12) make it possible to re-examine the relationship between dietary fat and certain aspects of cholesterol metabolism.

In order to define some of the mechanisms by which dietary factors regulate the excretion of neutral sterols, the effects of several fatty acids on cholesterol and coprostanol excretion have been studied in the rat. This communication presents evidence that linoleic acid accelerates coprostanol formation and that palmitic and oleic acids inhibit it.

EXPERIMENTAL

Treatment of Animals. Male rats of the Long-Evans strain, weighing 100 to 200 g, were fasted for 24 hours, then placed in individual cages, and fed diets which either were fat-free or contained fatty acids at levels which varied from 4% to 20%.[1] Four days after beginning the experimental diets the rats were anesthetized with ethyl ether, and a solution of cholesterol-4-C^{14} in Tween 20, containing 1.12×10^6 cpm, was injected into the tail vein (13). Feces were subsequently collected from each rat for periods of 2 to 4 days. At the end of the experimental period the rats were killed, and the intestines were removed in one piece and frozen at −20° until analyzed.

METHODS

The samples of feces were dried in an evacuated desiccator for 24 hours, weighed, and pulverized with a mortar and pestle. The powder was transferred with 100 ml of chloroform:methanol 3:1 (v/v) into an Erlenmeyer flask. The lipids were extracted by heating the slurry to boiling. The lipid extract was filtered and dried by warming under a stream of nitrogen. Ten milliliters of 20% potassium hydroxide in ethanol was then added; the samples were gassed with nitrogen, capped, and allowed to sit at room temperature for 90

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† Established Investigator of the American Heart Association.

[1] Each 1,000 g of the fat-free diet contained 250 g of casein, 680 g of glucose, 70 g of Hawk-Oser Salt Mix, 1 bottle Folbesyn® 10 mg folic acid, 0.5 g inositol, 0.1 ml Oleum Percomorphum®. In the diets containing 4% or 5% fatty acids, the acids were added directly to this mixture. In order to keep the protein content similar, each 1,000 g of the 20% fatty acid diets contained 250 g casein, 480 g glucose, 200 g fatty acid, and salts and vitamins as before.

minutes. At the end of the saponification period, 10 ml of water was added, and the solution was extracted twice with 100 ml of petroleum ether by shaking on an International bottle shaker #2 for 5 minutes. The petroleum ether extracts were evaporated to about 50 ml and washed first with 5 ml of 1 N sodium hydroxide in 50% ethanol and then with 5 ml of 50% ethanol. The washed petroleum ether fraction was dried under nitrogen and dissolved in 2 ml of chloroform. One portion was analyzed by gas-liquid chromatography as described below.

The intestines were divided into appropriate segments. The individual segments and their contents were added directly to 20% potassium hydroxide in ethanol and saponified for 90 minutes, following which the neutral sterols of the intestine were analyzed by the same procedure used for the fecal sterols.

The sterols were chromatographed by the method of VandenHeuvel *et al.* (11). Five- to 10 μl-portions of the chloroform solutions were injected into a Research Specialities Company gas-liquid chromatography assembly equipped with an ionization detector. Six-foot × 4-mm columns packed with 3.5% SE 30 silicone on Chromosorb W, 60 to 80 mesh, were used in these experiments, and the operating temperatures were as follows: column, 230°; detector, 250°; outlet, 300°; and vaporizer, 310°. The argon inlet pressure was 30 psi, and the monitored outflow was 100 ml/minute. The areas recorded under the various peaks were estimated by triangulation.

In some experiments the sterols were collected at the outlet in glass tubes filled with cotton. The materials were eluted from the cotton with petroleum ether, dried, and treated in one of two ways. Some samples were chromatographed on paper using the reversed phase system described by Martin (14); the spots were developed by spraying with either 8% phosphomolybdic acid or phosphotungstic acid in ethanol. Digitonides were precipitated and assayed as described by Sperry and Webb (15). Other samples were added to 10 ml of 0.4% diphenyloxazole in toluene, and assayed for C^{14} in a liquid scintillation counter.

MATERIALS

Cholesterol-4-C^{14} was purchased from the Chicago Nuclear Corporation, Chicago, Illinois; linoleic acid from the Hormel Foundation, Austin, Minnesota; and oleic acid from the J. T. Baker Chemical Company, Phillipsburg, New Jersey; SE 30 silicone was supplied by the General Electric Company, Silicone Products Department, Waterford, New York.

RESULTS

Separation and Quantification of Fecal Sterols. The utilization of the silicone-Celite column described by VandenHeuvel *et al.* (11) was found to provide a satisfactory separation of coprostanol from cholesterol (Fig. 1). Although variations in the silicone content

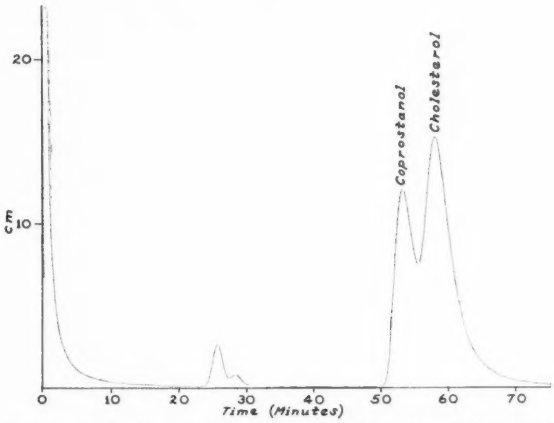

FIG. 1. Separation by gas-liquid chromatography of cholesterol and coprostanol; 10 μg of cholesterol and 10 μg of coprostanol in 10 μl of chloroform were injected into a gas-liquid chromatographic assembly. Conditions: 3.5% SE 30 silicone gum on Chromosorb W (60 to 80 mesh); 6-foot × 4-mm column; 230°; argon flow, 100 ml/minute; sensitivity 10. Two small contaminants of the coprostanol can be seen at 24 and 28 minutes.

of the column packing material from 0.5% to 5%, and in the column operating temperature from 210° to 250°, markedly altered the appearance time of cholesterol, the relative retention time of coprostanol to cholesterol (0.9) remained constant. The operating temperatures and silicone concentrations used in these experiments were chosen because they provided convenient and clear-cut separation of cholesterol and coprostanol from the many other substances present in extracts of biological materials.

Despite the fact that the peaks for coprostanol (appearance time 48 to 50 minutes) and cholesterol (appearance time 54 to 56 minutes) overlap in the system used here, a linear relationship was demonstrated between the amounts of these materials injected and the area recorded under the curve for quantities ranging from 5 μg to 50 μg (Fig. 2). Of the sterols tested in this system (desmosterol, cholestanol, cholestanone, 7-dehydrocholesterol, cholestenone, epicholesterol, epicoprostanol, methostenol, stigmasterol, and sitosterol), the only peaks which superimposed were those of cholestanol and cholesterol. No other significant contamination of either cholesterol or coprostanol could be demonstrated. The quantity of cholestanol in

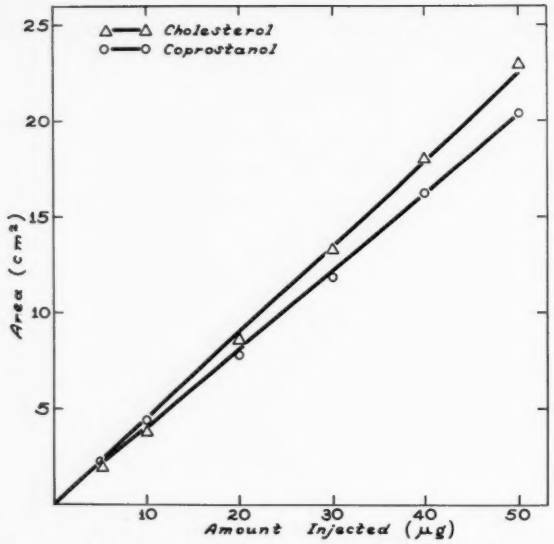

FIG. 2. Relation of varying quantities of cholesterol and coprostanol to area recorded. Varying amounts of cholesterol and coprostanol in 5 μl of chloroform were injected into a gas-liquid chromatography assembly. Conditions as in Figure 1.

feces was in no case sufficient to be detected by the paper chromatographic system of Martin (14), and the degree of contamination of the cholesterol in these experiments was therefore negligible. The fecal sterols found in largest quantities were cholesterol and coprostanol.

Effect of Linoleic and Palmitic Acids. Marked differences in neutral sterol excretion were noted when rats were fed either linoleic or palmitic acid. The addition of linoleic acid to the diet resulted in the excretion of large amounts of coprostanol, whereas the feeding of palmitic acid caused cholesterol to be the predominant sterol in the feces. These effects are demonstrated in the representative chromatograms shown in Figure 3. In Table 1 (experiment 1), the effects on neutral sterol excretion of supplementing the fat-free diet with either palmitic acid or linoleic acid are summarized. There was little difference among the three groups of rats, either in weight or in the average daily caloric intake. When compared to that of the fat-free group, the average weight of the dried feces was slightly increased in the linoleic acid group, and markedly increased (threefold) in the animals fed palmitic acid.

The most striking differences among the three groups of animals were in the partition of the neutral sterols. The animals fed the fat-free diets excreted an average of 2.2 mg coprostanol and 1.0 mg cholesterol per day. The linoleic acid-fed animals excreted an average of 6.2 mg coprostanol and 4.2 mg cholesterol daily, while the rats fed palmitic acid excreted an average of 1.2 mg coprostanol and 4.0 mg cholesterol per day. The suppression of coprostanol excretion by palmitic acid is particularly noteworthy since it occurred under circumstances in which total neutral sterol excretion was unchanged.

Effect of Linoleic and Oleic Acids. Since palmitic acid has an exceedingly low coefficient of digestibility, coprostanol formation was studied in rats fed smaller amounts of linoleic and oleic acids, fatty acids which have similar coefficients of digestibility (16). Representative chromatograms of fecal sterols from these rats are shown in Figure 4. Once again, the rats fed either no fat or linoleic acid excreted large quantities of coprostanol, whereas the rat fed oleic acid excreted cholesterol predominantly. The results of feeding isocaloric quantities of diets containing these acids to 14 rats are summarized in Table 1 (experiment 2). There were no significant differences in body weight, average daily caloric intake, or weight of the dried feces. Further-

TABLE 1. EFFECT OF FATTY ACIDS ON COPROSTANOL EXCRETION BY THE RAT

Exp.	Diet	Number of Rats	Average Weight	Average Daily Food Intake		Average Daily Weight of Dried Feces	Sterol* Excretion/24 Hours		
							Coprostanol	Cholesterol	Total
			g	*g*	*cal*	*g*	*mg*	*mg*	*mg*
1†	Fat free	5	153	6.8	23.2	0.3	2.2 ± 1.1	1.0 ± 0.2	3.2 ± 1.7
	20% linoleic acid	5	132	4.6	21.5	0.4	6.2 ± 1.6	4.2 ± 2.4	10.4 ± 3.1
	20% palmitic acid	5	149	5.2	24.4	1.0	1.2 ± 0.8	4.0 ± 1.4	5.2 ± 1.8
2‡	Fat free	5	153	10	38	0.6	2.4 ± 1.8	4.9 ± 2.7	7.3 ± 1.7
	5% oleic acid	4	150	10	40	0.5	1.6 ± 0.7	8.9 ± 3.3	10.5 ± 2.7
	5% linoleic acid	5	160	10	40	0.7	7.8 ± 1.4	2.8 ± 1.2	10.6 ± 2.3

* Sterols separated and chromatographed as described in the text.

† Feces collected during the last 4 days of an 8-day experimental period.

‡ Feces collected during the last 2 days of a 6-day experimental period.

more, there were no consistent differences in total sterol excretion between the three groups. However, again there were marked differences in the average daily coprostanol excretion (fat-free, 2.4 mg; linoleic acid, 7.8 mg; oleic acid, 1.6 mg) and in the average daily cholesterol excretion (fat-free, 4.9 mg; linoleic acid, 2.8 mg; oleic acid, 8.9 mg).

Effect of Mixtures of Linoleic and Oleic Acids. Coprostanol excretion was then studied in rats fed varying amounts of linoleic and oleic acids. The results of feeding these diets to 23 animals are summarized in Table 2. No consistent differences in body weight, average daily caloric intake, weight of the dried feces, or total sterol excretion were observed among the five groups. Again there were marked differences in the average daily coprostanol excretion between the rats fed 4% linoleic acid (5.1 mg) and those fed 4% oleic acid (0.5 mg). It was found that the addition of 0.1% linoleic acid to the 4% oleic acid diet did not increase coprostanol excretion. Furthermore, the addition of 2% linoleic acid to an equal amount of oleic acid also did not elevate fecal coprostanol. Thus, in the presence of oleic acid, linoleic acid does not seem capable of accelerating coprostanol excretion.

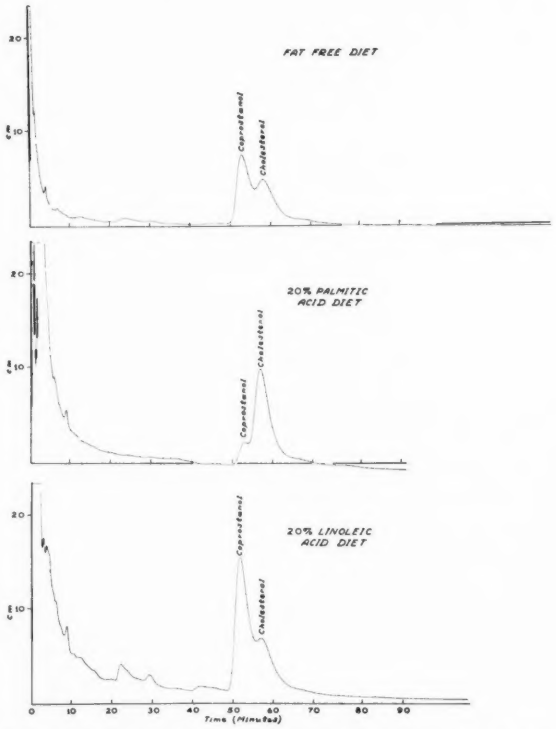

FIG. 3. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained either no fat, 20% palmitic acid, or 20% linoleic acid. Five-microliter aliquots of the chloroform extracts were chromatographed under the same conditions described in Figure 1. The peaks labeled cholesterol were identified by comparison of the appearance time in this system (56 minutes) with that of authentic cholesterol (56 minutes). In addition, the material was collected, and an aliquot was rechromatographed in *n*-propanol-kerosene (14) (R_f of unknown, 0.48; R_f of cholesterol, 0.48). The peaks labeled coprostanol were identified by comparison of the appearance time in this system (48 minutes) with that of authentic coprostanol (48 minutes). The migrations of the collected material were also compared in the *n*-propanol-kerosene system (R_f of unknown, 0.34; R_f of coprostanol, 0.35); both the coprostanol and cholesterol gave a blue reaction when sprayed with the phosphomolybdic reagent; cholesterol gave a red color when developed in phosphotungstic acid, and coprostanol did not react at all. The material labeled coprostanol was digitonin precipitable and did not react with Liebermann-Burchard reagent (15).

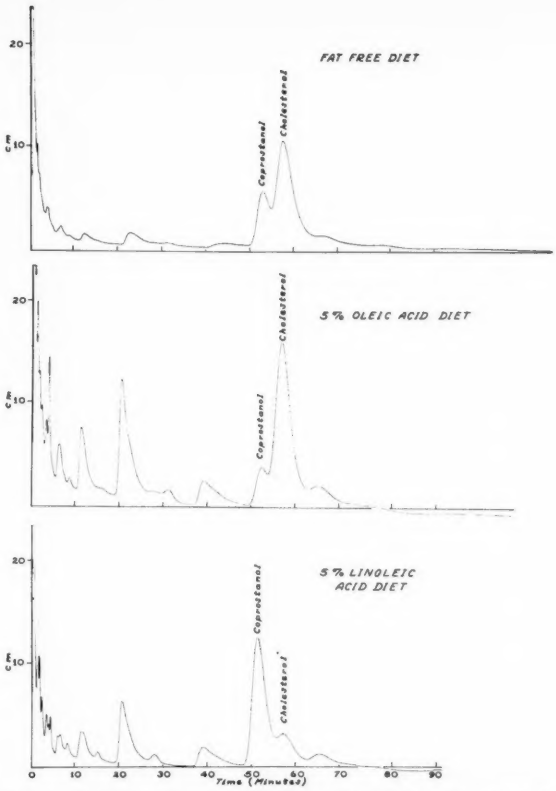

FIG. 4. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained either no fat, 5% oleic acid, or 5% linoleic acid. Five-microliter aliquots of the chloroform extracts were chromatographed as described in Figure 1. The identification of the peaks is described in Figure 3.

Influence of Linoleic and Oleic Acids on the Neutral Excretory Products of Cholesterol-4-C^{14}. In order to determine which of the fecal sterols in these experiments were derived from the exchangeable cholesterol pool of the rat, C^{14}-labeled cholesterol was given intravenously to the rats described in the previous section.

TABLE 2. EFFECT OF VARYING AMOUNTS OF LINOLEIC ACID AND OLEIC ACID ON COPROSTANOL EXCRETION BY THE RAT

	Number of Rats	Average Weight	Average Daily Food Intake		Average Daily Weight of Dried Feces	Sterol Excretion/24 Hours		
						Coprostanol	Cholesterol	Total
		g	*g*	*cal*	*g*	*mg*	*mg*	*mg*
Fat free	5	130	14	53	0.5	3.5 ± 1.5	2.8 ± 1.1	6.3 ± 1.3
4% linoleic acid	4	123	12	47	0.5	5.1 ± 0.5	2.0 ± 0.2	7.1 ± 0.6
2% oleic acid-2% linoleic acid	5	142	14	55	0.6	0.9 ± 0.4	5.4 ± 2.9	6.3 ± 2.7
4% oleic acid-0.1% linoleic acid	5	148	14	55	0.5	0.5 ± 0.4	4.9 ± 0.9	5.4 ± 1.0
4% oleic acid	4	143	14	55	0.6	0.5 ± 0.5	6.5 ± 1.6	7.0 ± 1.2

Four days after starting the experimental diets, 0.4 μmole cholesterol containing 1.12×10^6 cpm cholesterol-4-C^{14} was injected into the tail vein. Feces were collected from the tenth through the fourteenth day. Sterols were separated, chromatographed, and assayed for C^{14} as described in the text.

As shown in Figure 5, when the various chromatographic peaks from representative rats from each of these groups were collected and assayed for C^{14}, the only areas containing significant radioactivity corresponded to cholesterol and coprostanol. Thus, in these experiments cholesterol and coprostanol were the only significant neutral excretion products of cholesterol.

Coprostanol Formation in the Intestine. In order to determine the site of coprostanol formation in these experiments, the intestines from three of the rats reported in Table 1 (experiment 2) were removed, and the coprostanol was measured in various portions of the gastrointestinal tract (Table 3). No coprostanol was found in the entire intestine of the rats fed the fat-free or 5% oleic acid diets. However, a small quantity of coprostanol was found in the ileum of the linoleic acid-fed animals, but in the contents and wall of the colon of this animal, coprostanol accounted for nearly half of the total neutral sterol. Furthermore, the coprostanol present in the large intestine (7.0 mg) corresponded closely to the excretion of coprostanol for the previous 2 days (10.4 mg/day). It was concluded, therefore, that the marked enhancement by linoleic acid of the bacterial reduction of cholesterol to coprostanol occurs in the lower intestinal tract.

DISCUSSION

It has been established both by studies *in vivo* and *in vitro* that the *cis* hydrogenation of cholesterol to form coprostanol in the intact animal is due to microbial action in the large intestine. No coprostanol is excreted by germ-free animals (17), and coprostanol formation can also be abolished by the administration of a number of bacteriostatic agents (18, 19, 20). In addition, the conversion of cholesterol to coprostanol by bacterial cultures isolated from feces has also been demonstrated conclusively (21 to 24), and the formation of coprostanol has been shown by the isotopic studies of Rosenfeld *et al.* (22) to be a direct microbial reduction, not involving a 3-one intermediate.

The factors determining the extent to which cholesterol is converted to coprostanol by the intestinal bacteria are poorly understood. Several studies have appeared reporting the influence of various dietary constituents on the synthesis of coprostanol. Milk diets (25), dietary lactose (26), sodium taurocholate (27), and Tween 80 (27) all markedly inhibit coprostanol formation. On the other hand, the early observations of Dorée and Gardner (28) suggested that the presence of brain in the diet accelerated coprostanol excretion. These observations were extended by Rosenheim and

TABLE 3. EFFECT OF LINOLEIC ACID AND OLEIC ACID ON COPROSTANOL FORMATION IN THE RAT INTESTINE

Intestinal Segment	Fat-Free Diet		5% Oleic Acid Diet		5% Linoleic Acid Diet	
	Coprostanol	Cholesterol	Coprostanol	Cholesterol	Coprostanol	Cholesterol
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Stomach	0	3.75	0	4.46	0	7.16
Duodenum	0	3.11	0	2.97	0	3.62
Jejunum	0	8.24	0	12.57	0	6.54
Ileum	0	8.10	0	10.14	0.1	11.22
Colon	0	11.89	0	16.76	7.0	8.27

Intestines from the rats reported in Table 2 were removed in one piece at the time of death and immediately frozen at −20°. The intestines were subsequently sectioned, and the intestinal segments plus contents were saponified as a unit; the sterols were extracted and assayed as described in the text.

Webster (29, 30), who concluded that the glycolipid phrenosin, obtained from brain and other organs, was an essential factor both for the formation of coprostanol and of coprositosterol. Wells (27), however, has reported that coprostanol excretion is unimpaired in rats fed purified synthetic diets for long periods of time.

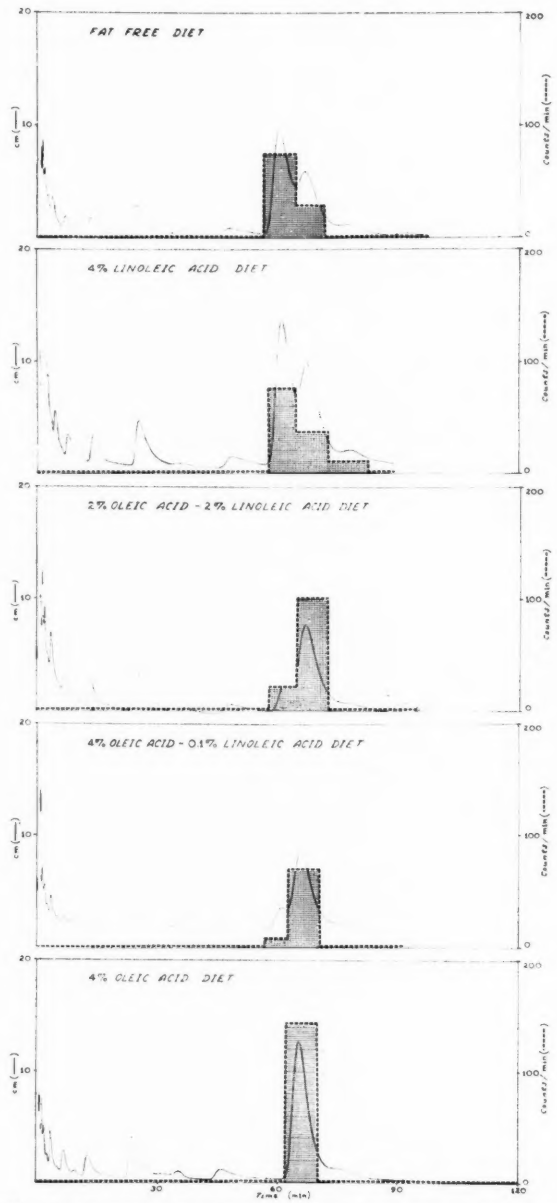

FIG. 5. Separation by gas-liquid chromatography of fecal sterols from rats fed diets which contained varying amounts of oleic and linoleic acids. The chromatographic conditions were as in Figure 1, except that the argon flow was 60 ml/minute; identification of the peaks is described in Figure 3. Each peak was collected in a sample-collecting tube and assayed for C^{14} as described in the text. The only peaks containing significant radioactivity were coprostanol (appearance time, 54 minutes) and cholesterol (appearance time, 62 minutes).

While the data reported here confirm the fact that the animals fed purified or even fat-free diets can synthesize coprostanol, far more striking is the demonstration that the presence of linoleic acid markedly accelerates the formation and excretion of coprostanol. In fact, this effect is of such a magnitude that in the animals fed linoleic acid, coprostanol was the major neutral sterol present in the feces. This enhancement of coprostanol formation by linoleic acid was even more marked when compared with the coprostanol excretion of rats fed diets containing either oleic or palmitic acids, both of which suppressed coprostanol formation. It is possible that the results previously reported by Wells and by Rosenheim and Webster might, in fact, be due to the fat content of the diets employed. The diets in the studies of Wells and Cooper (26, 27) were all supplemented with either methyl linoleate, cod liver oil, or corn oil, and Rosenheim and Webster (29) observed that the brain extracts did not accelerate coprostanol formation in the absence of dietary fat.

The present studies do not furnish evidence as to the mechanism by which linoleic, palmitic, and oleic acids influence coprostanol formation. It would seem unlikely that the effect of linoleic acid is due solely to its action as an essential fatty acid, for although the feeding of saturated fat accelerates the development of essential fatty acid deficiency, oleic acid (in the form of triolein) neither accelerates nor retards the development of this condition (30). Attempts to clarify the mechanisms of these effects are now in progress.

Further studies will likewise be necessary before it is possible to draw any conclusions regarding a possible relationship between the acceleration of coprostanol formation by linoleic acid and the previously demonstrated cholesterol-lowering effect of linoleic acid both in man (12) and the hypercholesterolemic rat (5).

The methostenol was a gift from Dr. W. W. Wells. The author is indebted to Joanne Sherwood and George T. Crowley for technical assistance.

REFERENCES

1. Kinsell, L. W., J. Partridge, L. Boling, S. Margen, and G. Michaels. *J. Clin. Endocrinol. and Metabolism* **12**: 909, 1952.
2. Ahrens, E. H., Jr., W. Insull, Jr., R. Blomstrand, J. Hirsch, T. Tsaltas, and M. L. Peterson. *Lancet* **1**: 943, 1957.

3. Malmros, H., and G. Wigand. *Lancet* **2:** 1, 1957.
4. Lambert, G. F., J. P. Miller, R. T. Olsen, and D. V. Frost. *Proc. Soc. Exptl. Biol. Med.* **97:** 544, 1958.
5. Hauge, J. G., and R. Nicolaysen. *Acta Physiol. Scand.* **45:** 26, 1959.
6. Wilson, J. D., and M. D. Siperstein. *Proc. Soc. Exptl. Biol. Med.* **99:** 113, 1958.
7. Wilson, J. D., and M. D. Siperstein. *Am. J. Physiol.* **196:** 596, 1959
8. Hellman, L., R. S. Rosenfeld, W. Insull, Jr., and E. H. Ahrens, Jr. *J. Clin. Invest.* **36:** 898, 1957.
9. Goldsmith, G. A., J. G. Hamilton and O. N. Miller. *Trans. Assoc. Am. Physicians* **72:** 207, 1959.
10. Fieser, L. F., and M. Fieser. *Steroids.* New York, Reinhold Publishing Corp., 1959, pp. 346–52.
11. VandenHeuvel, W. J. A., C. C. Sweeley and E. C. Horning. *J. Am. Chem. Soc.* **82:** 3481, 1960.
12. Kinsell, L. W., G. D. Michaels, R. W. Friskey, and S. Splitter. *Lancet* **1:** 334, 1958.
13. Meier, J. R., M. D. Siperstein and I. L. Chaikoff. *J. Biol. Chem.* **198:** 105, 1952.
14. Martin, R. P. *Biochim. et Biophys. Acta* **25:** 408, 1957.
15. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
16. Hoagland, R., and G. G. Snider. *J. Nutrition* **26:** 219, 1943.
17. Danielsson, H., and B. Gustafsson. *Arch. Biochem. Biophys.* **83:** 482, 1959.
18. Rosenheim, O., and T. A. Webster. *Biochem. J.* **37:** 580, 1943.
19. Coleman, D. L., and C. A. Baumann. *Arch. Biochem. Biophys.* **66:** 226, 1957.
20. Wainfan, E., G. Henkin, L. I. Rice, and W. Marx. *Arch. Biochem. Biophys.* **38:** 187, 1952.
21. Dam, H. *Biochem. J.* **28:** 820, 1934.
22. Rosenfeld, R. S., D. K. Fukushima, L. Hellman, and T. F. Gallagher. *J. Biol. Chem.* **211:** 301, 1954.
23. Snog-Kjaer, A., I. Prange and H. Dam. *J. Gen. Microbiol.* **14:** 256, 1956.
24. Coleman, D. L., and C. A. Baumann. *Arch. Biochem. Biophys.* **72:** 219, 1957.
25. Gamble, J. L., and K. D. Blackfan. *J. Biol. Chem.* **42:** 401, 1920.
26. Wells, W. W., and S. B. Cooper. *Arch. Biochem. Biophys.* **75:** 273, 1958.
27. Wells, W. W. *Arch. Biochem. Biophys.* **66:** 217, 1957.
28. Dorée, C., and J. A. Gardner. *Proc. Roy. Soc. (London)* **B80:** 227, 1908.
29. Rosenheim, O., and T. A. Webster. *Biochem. J.* **35:** 920, 1941.
30. Rosenheim, O., and T. A. Webster. *Biochem. J.* **35:** 928, 1941.

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The use of cholesterol-4-C^{14}-labeled lipoproteins as a tracer for plasma cholesterol in the dog*

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SUMMARY

A method is described for labeling lipoprotein cholesterol with cholesterol-4-C^{14}. The specific activity of the labeled cholesterol is equal in low and high density lipoproteins, and none of the label is associated with other serum proteins. Lipoproteins labeled in this manner were used to define the quantity of cholesterol that exchanges with plasma free cholesterol in 30 hours. This quantity is approximately three times the amount of cholesterol contained in blood plasma, red blood cells, and liver.

Study of the fraction of total body cholesterol that is in equilibrium with cholesterol in blood plasma has been hampered by lack of a tracer whose behavior is identical with that of plasma cholesterol. Since cholesterol exists in blood plasma as a constituent of lipoproteins, artificial cholesterol emulsions are not suitable for this purpose. Emulsified or suspended cholesterol labeled with C^{14} is rapidly removed from the circulation, and reappears later as labeled free and ester cholesterol associated with lipoproteins (1, 2, 3).

In 1955 Eckles and her collaborators (4) demonstrated the rapid exchange of isotopically labeled free cholesterol between plasma, liver, and red blood cells. This observation led to the concept of a rapidly exchangeable liver-blood pool of cholesterol. The limits of this pool have not been determined as yet, nor has its physiologic significance been evaluated.

This paper describes a method of incorporating isotopically labeled free cholesterol into plasma lipoproteins *in vitro* and the utilization of this material to study the kinetics of the early phases of plasma free cholesterol turnover in the dog.

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METHODS

Incorporation of Cholesterol into Lipoproteins. Two to five microcuries of cholesterol-4-C^{14} (specific activity 28 μc/mg)[1] was dissolved in 1 ml of absolute ethanol. The solution was rapidly injected from a syringe through a 23-gauge needle under the surface of 10 ml of a 5% solution of human serum albumin in 0.15 M NaCl. The mixture was allowed to stand for 20 minutes. Alcohol was then evaporated from the solution at 25° under an air stream. Volume was maintained at 10 ml by frequent small additions of distilled water. When the odor of alcohol could no longer be detected, the solution was mixed with 10 ml of whole blood serum from the experimental animal, and allowed to stand for 1 hour. The protein-free density of the mixture was raised to 1.019 by the addition of a saline solution of density 1.083. The solution was centrifuged for approximately 15 hours at 4° in the 40.3 rotor of a Spinco Model L ultracentrifuge at 109,000 × g. The supernatant material was removed with a tube slicer (5) and discarded. The sedimented material was redispersed prior to use.

Administration of Labeled Lipoproteins. One male and one female mongrel dog, maintained on a diet of nine parts Friskies dog meal and one part lean horse meat, were used. A measured amount (15 to 20 ml) of labeled solution was rapidly injected from a calibrated syringe into a foreleg vein. Samples were withdrawn at timed intervals from a vein in the opposite foreleg.

[1] Obtained commercially from Nuclear-Chicago Corporation, Chicago, Ill.

Food was withheld for 16 hours before and 48 hours after the injection; access to water was allowed at all times. In dog #1 red blood cell and plasma volumes (6, 7) were determined one week later. At this time both animals were sacrificed. The liver of each was back-perfused through the vena cava with physiologic saline, blotted and weighed.

Analytical Procedures. Blood specimens were collected in tubes containing 1.5 mg sodium oxalate per ml, mixed, and chilled immediately in ice water. Within 15 minutes the samples were centrifuged at 500 × *g* for 20 minutes at 4°. The plasma was removed and stored at 4°. The red cells were washed three times with 25 volumes of ice-cold physiologic saline solution, and centrifuged at 18,000 × *g* for 15 minutes in the SW-39 rotor of a Spinco Model L ultracentrifuge. The plastic tubes were cut with a tube slicer just below the top of the packed red blood cells. One-milliliter aliquots of the cells were drawn into a calibrated syringe through a large bore needle, discharged into tubes containing 2 ml of distilled water, mixed to achieve hemolysis, and stored at 4°.

The lipids were extracted from 2 ml of plasma or hemolyzed red blood cells in 50 ml of alcohol-acetone 1:1 (v/v). Liver lipids were extracted similarly in a Waring Blendor. Aliquots of the extracts were taken for determination of free and total cholesterol in plasma and liver and for free cholesterol in red blood cells[2] (8). The supernatant fluids and washings from the free cholesterol digitonide precipitates were saved. Duplicate aliquots of free cholesterol digitonides were prepared (8) and dissolved in 1.2 ml of methanol. One milliliter of the methanolic solution was counted in a Packard Tri-Carb liquid scintillation spectrometer, with 10 ml of 0.3% diphenyloxazole (PPO) in toluene as phosphor. No quenching correction was used, since quenching was found to be equal in all samples. The supernatant fluids and washings from the free cholesterol digitonide precipitates were pooled, taken to dryness under an air stream, and extracted three times with 4 ml of heptane. The heptane was evaporated and the residue dissolved in 10 ml of 0.3% PPO in toluene for counting.

Distribution of Labeled Cholesterol in Lipoproteins. To define the nature of the binding of the labeled material *in vitro* and *in vivo*, selected samples of the material to be injected and of the blood serum after injection were separated into lipoprotein classes at densities 1.019, 1.063, and 1.21, according to the method of Havel *et al.* (9). The lipids were extracted from these fractions and analyzed as described for whole blood plasma.

Calculations. In dog #1 the total amounts of free and ester cholesterol in blood were computed by multiplying the determined red cell and plasma volumes by the concentrations of plasma free and ester cholesterol and red blood cell free cholesterol. In dog #2 plasma and red blood cell volumes were estimated on the basis of the volumes determined in dog #1. These estimated figures were used in similar computations.

[2] Preliminary work with red blood cells demonstrated that this method of extraction gives the same results as a 24-hour extraction with alcohol-acetone in a Soxhlet apparatus.

RESULTS

Lipoprotein Incorporation. After an incubation period of 1 hour the labeled serum was adjusted to a density of 1.063 and centrifuged for 1 hour at 109,000 × *g*. The tubes were sliced so that the top fraction contained approximately one-third the total volume. Under these conditions there is little stratification of cholesterol-containing lipoproteins in the tube. The specific activities of the top and bottom fractions isolated from the tubes were approximately equal (Table 1), demonstrating that the labeled cholesterol was not concentrated in any one layer in the tube. Further centrifugation at this same density for 16 hours separated the high and low density lipoproteins. The specific activities of the two fractions were practically equal, despite marked differences in cholesterol concentrations (Table 1).

Further fractionation (Table 2) showed no detectable C^{14}-labeled cholesterol in proteins of d > 1.21, and approximately equal specific activities in the d =

TABLE 1. DISTRIBUTION AND SPECIFIC ACTIVITY OF FREE CHOLESTEROL IN LIPOPROTEIN FRACTIONS SEPARATED AT DENSITY 1.063

Sample	Centrifugation Time	Fraction	Free Cholesterol	
			Distribution	Specific Activity
	hours		*mg/fraction*	*cpm/mg*
1	1	Top	0.45	33,000
		Bottom	0.68	36,100
	16	Top (low density)	0.23	34,700
		Bottom (high density)	0.86	32,400
2	16	Top (low density)	0.38	20,300
		Bottom (high density)	0.61	20,400

TABLE 2. DISTRIBUTION AND SPECIFIC ACTIVITY OF FREE CHOLESTEROL IN LIPOPROTEIN FRACTIONS

Fraction	Free Cholesterol		
	Distribution	Radioactivity	
	mg/100 ml	*cpm/100 ml*	*cpm/mg*
$d < 1.019$	*	1,290	*
$d = 1.019$ to 1.063	16	17,100	1,140
$d = 1.063$ to 1.21	27	32,300	1,200
$d > 1.21$	0	0	0
Whole serum	45	50,800	1,130

* Fraction contained too little cholesterol for accurate measurement.

1.063 to 1.21 and d = 1.019 to 1.063 fractions. Fasting dog plasma contains so little lipoprotein material of $d < 1.019$ that specific activities could not be determined accurately, but less than 2% of total plasma radioactivity was present in this fraction. Because the specific activity of this fraction may have been higher than that of the other two fractions, material for injection was first centrifuged at d = 1.019, after which the supernatant material was discarded. The incorporation of cholesterol-4-C^{14} into lipoproteins varied from 40% to 80%. Most of the loss was probably attributable to adherence of the labeled material to glass or plastic tubes, particularly during alcohol evaporation.

In Vivo Behavior of Labeled Lipoprotein. Table 3 summarizes pertinent data on both dogs. Figure 1 shows the specific activity-time curve of blood cholesterol fractions in dog #1, plotted to give the best visual fit. A similar curve was drawn for dog #2. In the case of dog #2, there were only three points beyond 24 hours, so that the slope of the terminal portion of the curve is subject to appreciable error. To allow comparison of the two sets of values, the slowest component of the curve was extrapolated to the ordinate, and this point was assigned a value of one. Figure 2 presents the data on both dogs, with the cholesterol specific activities expressed as a fraction of this intercept. The values for plasma free cholesterol in the two animals appeared to fall on the same line. Resolution of the entire free cholesterol curve by successive subtraction of each semilogarithmic component gave five exponential terms with half times of 4 minutes, 30 minutes, 65 minutes, 7 hours, and 96 hours (Fig. 3). Because of the few experimental points beyond 30 hours, the assumption that all the data fall on the same curve is somewhat arbitrary. However, the large number of points in close agreement prior to 30 hours prompted the use of this method of analyzing the data.

As shown in Figures 1 and 2, the red cell free, plasma free, and plasma ester cholesterol reached equilibrium in about 30 hours. The quantity of cholesterol which apparently equilibrated in 30 hours was arbitrarily designated C_e. This value was computed by dividing the quantity of injected radioactivity by the intercept of the slowest component of the curve for each animal. The measured quantity in the anatomic blood-liver pool was found to account for less than one-half of C_e (Table 4).

In two sera from each dog, taken 3 and 5 hours after injection and separated into lipoprotein fractions, virtually no activity was found in the fraction $d > 1.21$. The specific activity of free cholesterol in the fraction d = 1.063 to 1.21 was the same as that of free cholesterol in whole plasma.

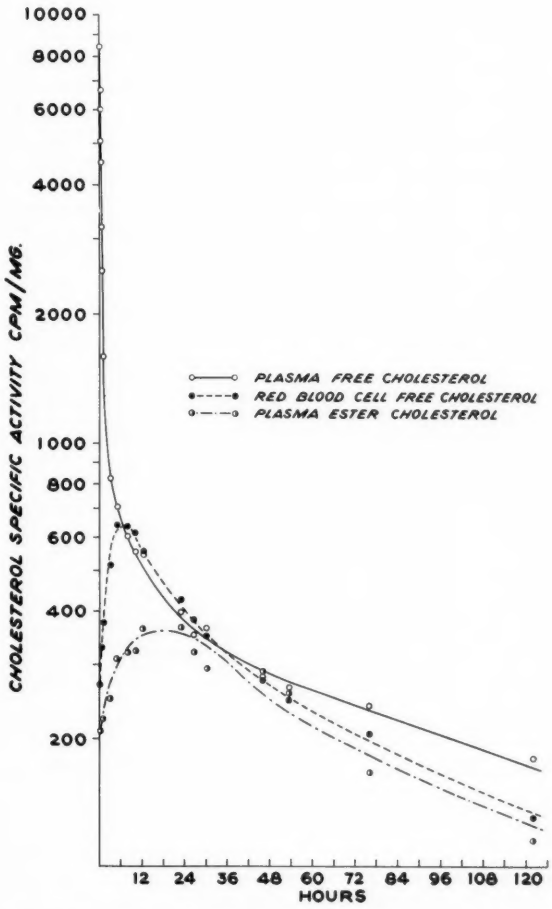

FIG. 1. Specific activities of blood cholesterol fractions after intravenous administration of lipoprotein-bound free cholesterol-4-C^{14} in dog #1.

TABLE 3. QUANTITY OF CHOLESTEROL IN PLASMA, RED BLOOD CELLS, AND LIVER OF DOGS

	Dog #1 (25.6 Kg)	Dog #2 (18.6 Kg)
Plasma		
Volume (ml)	1,100	800*
Free cholesterol (mg/100 ml)	30	56
Ester cholesterol (mg/100 ml)	73	136
Red blood cells		
Volume (ml)	688	500*
Free cholesterol (mg/100 ml)	157	172
Liver		
Weight (g)	760	590
Total cholesterol (mg/g)	2.08	2.45

* Estimated volume.

DISCUSSION

In Vitro Labeling. Because the precise location of cholesterol in lipoprotein molecules is not known, no absolute criterion is available for judging whether the material labeled by our method is identical with biosynthetically labeled free cholesterol. Several characteristics of the *in vivo* behavior of our labeled lipoprotein cholesterol strongly suggest that there is a close resemblance: (*a*) We found a smoothly curvilinear venous disappearance curve of labeled free cholesterol, whereas labeled cholesterol in emulsions characteristically disappears almost completely from the circulation within 1 hour and subsequently reappears, reaching maximal specific activity 24 to 48 hours after injection (1, 2, 3). Such behavior has been attributed to rapid phagocytosis of the emulsified lipid (1) and its subsequent slow release in lipoprotein form. (*b*) The injected cholesterol equilibrated rapidly with red cell free cholesterol. Hagerman and Gould (10), in studies using cholesterol labeled biosynthetically from C^{14}-acetate, reported a half time for this equilibrium of 70 minutes *in vitro*, which would result in 97% equilibration in slightly less than 6 hours. The average time for equilibration of plasma and red cell free cholesterol specific activities in our experiments was 5 hours, and graphical subtraction revealed a component with a half time of equilibration of 65 minutes (see below). (*c*) Specific activities in the major lipoprotein fractions were equal. Biggs and Nichols (11) and others (12) have shown that C^{14}-labeled free cholesterol in chylomicrons harvested from the thoracic duct of animals fed cholesterol-C^{14} rapidly equilibrates with unlabeled serum lipoprotein cholesterol during *in vitro* incubation.

TABLE 4. COMPONENTS OF EXCHANGEABLE CHOLESTEROL (C_e)

Cholesterol	Dog #1	Dog #2
	mg	*mg*
C_e	11,180	10,050
Plasma		
Free	330	450
Ester	800	1,090
Red blood cell	1,080	860
Liver	1,580	1,470
Blood-liver pool	3,790	3,870
C_e minus blood-liver pool	7,390	6,180

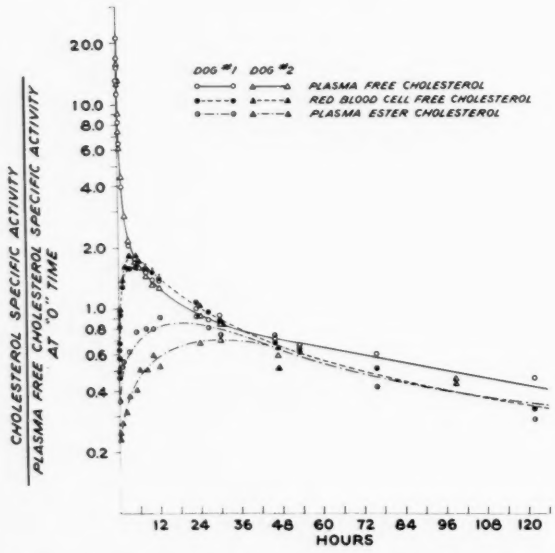

FIG. 2. Specific activities of blood cholesterol fractions after intravenous administration of lipoprotein-bound free cholesterol-4-C^{14} in two dogs. The extrapolated intercept of the slowest component of the plasma free cholesterol specific activity-time curve has been assigned a value of one.

Avigan (3) recently reported a similar method for incorporation of C^{14}-labeled sterols and steroids into lipoproteins; however, in contrast to our results, he found appreciable quantities of C^{14}-labeled material in the $d > 1.21$ fraction. In our studies the amount of C^{14}-labeled cholesterol taken up by low and high density lipoproteins was a function of the quantity of cholesterol already present, which suggests that the incorporation resulted from molecular exchange. The ease of introduction of the labeled material and its subsequent rapid interchange with red cell and liver cholesterol support the concept of a peripheral location for the free cholesterol normally contained in the lipoprotein molecule.

The ability to label lipoprotein cholesterol should permit more definitive studies of plasma cholesterol

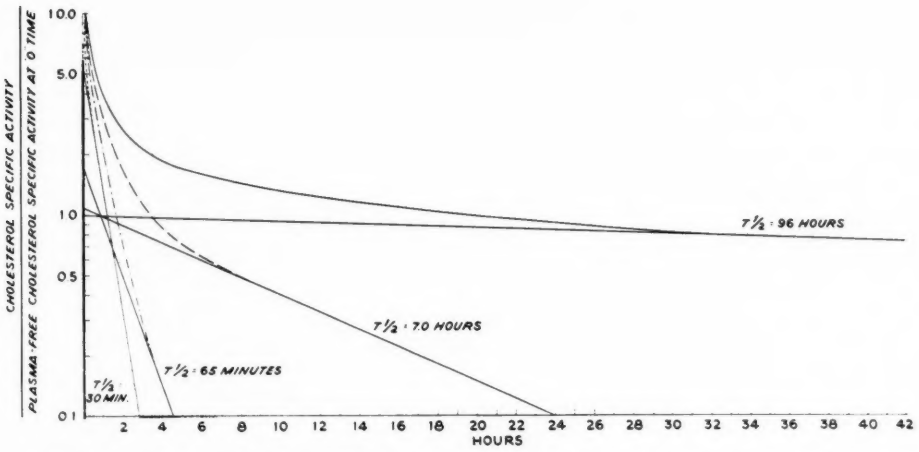
CHOLESTEROL SPECIFIC ACTIVITY
PLASMA FREE CHOLESTEROL SPECIFIC ACTIVITY AT 0 TIME
100
5.0
1.0
0.5
0.1
T½ : 96 HOURS
T½ : 7.0 HOURS
T½ : 65 MINUTES
T½ 30 MIN.
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
HOURS

FIG. 3. Graphic resolution of the specific activity-time curve of plasma free cholesterol in Figure 2.

metabolism, including pathways of conversion to ester cholesterol and mechanisms of excretion.

Venous Disappearance Curves. The exponential rate constants derived from the disappearance of labeled plasma free cholesterol must be interpreted with caution, as a small error in the determination of the slope of the slowest component of the curve can result in magnified errors in the other components. With this in mind, however, we have compared the rate constants we observed with cholesterol turnover times reported in the literature. The second rate constant (half time 30 minutes) corresponds most closely to that reported by Eckles *et al.* (4) for exchange between free cholesterol of liver and plasma in dogs. The third rate constant (half time 65 minutes) is almost identical with that reported for exchange of free cholesterol between plasma and red blood cells *in vitro* (10). It is likely, therefore, that these two exchange reactions are major determinants of the second and third rate constants. The fourth rate constant (half time 7 hours) must be determined in part by formation of plasma ester cholesterol, since its specific activity was approaching that of plasma free cholesterol during the first 30 hours. The slowest component (half time 96 hours) includes metabolic turnover plus other slowly equilibrating compartments. This is a somewhat faster turnover time than that reported previously in the dog (13), but complete equilibrium with total body cholesterol undoubtedly did not occur in 4 days. The most rapid component (half time 4 minutes) cannot be related to any known physiological compartment.

Three possibilities could account for the finding that C_e was considerably larger than the anatomic blood-liver pool. First, labeled cholesterol could be lost irreversibly by excretion or chemical alteration prior to 30 hours. We have calculated the extent to which such loss could contribute to C_e based on the following considerations. Maximum fecal excretion of endogenous sterols and of bile acids is said to be 22 mg (14) and 15 mg (15), respectively, per kilogram body weight per day in dogs, and the specific activity of these compounds could not have exceeded the mean specific activity of plasma free cholesterol during this period. In dog #1, which weighed 25.6 kg, the mean specific activity of plasma free cholesterol during the first 30 hours was 730 cpm/mg. Calculated excretion of sterols and bile acids during this period should not have exceeded 1180 mg ($[22 + 15] \times 25.6 \times 30/24$), so that not more than 0.86×10^6 cpm (1180×730) could have been excreted during this time. This is only about 20% of the quantity of radioactivity injected, and the actual amount probably was considerably less than this. Similar calculations lead to equivalent results in dog #2. Second, there may be anatomical sites other than red blood cells and liver in which equilibrium is complete in 30 hours. To our knowledge, such sites have not been described. Third, incomplete equilibration with cholesterol may take place in still other sites. Gould (16) administered C^{14}-acetate to dogs and injected their blood containing C^{14}-cholesterol into recipient animals. After 30 hours the specific activity of cholesterol of visceral organs and muscle and connective and adipose tissues was 30% to 50% that of plasma free cholesterol. Therefore the third possibility appears to offer the best explanation of the magnitude of C_e. It is clear that C_e does

not correspond to any anatomical compartment and includes cholesterol not in isotopic equilibrium at 30 hours.

Special note should be made of the fact that the specific activity of plasma ester cholesterol, despite its greater experimental variability, never exceeded that of plasma free cholesterol. This finding differs from those of previous studies performed after feeding isotopically labeled cholesterol or acetate or injecting labeled acetate (17, 18, 19). Our results, in agreement with those of other workers (20, 21), suggest that liver free cholesterol, which is in isotopic equilibrium with plasma free cholesterol, is not the only precursor of plasma ester cholesterol.

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REFERENCES

1. Hirsch, E. F., and S. Weinhouse. *A.M.A. Arch. Pathol.* **30:** 1079, 1940.
2. Portman, O. W., and L. Sinisterra. *J. Exptl. Med.* **106:** 727, 1957.
3. Avigan, J. *J. Biol. Chem.* **234:** 787, 1959.
4. Eckles, N. E., C. B. Taylor, D. J. Campbell, and R. G. Gould. *J. Lab. Clin. Med.* **46:** 359, 1955.
5. Randolph, M. L., and R. R. Ryan. *Science* **112:** 528, 1950.
6. Nomof, N., J. Hopper, Jr., E. Brown, K. Scott, and R. Wennesland. *J Clin. Invest.* **33:** 1382, 1954.
7. Gibson, J. G., and K. A. Evelyn. *J. Clin. Invest.* **17:** 153, 1938.
8. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
9. Havel, R. J., H. A. Eder and J. H. Bragdon. *J. Clin. Invest.* **34:** 1345, 1955.
10. Hagerman, J. S., and R. G. Gould. *Proc. Soc. Exptl. Biol. Med.* **78:** 329, 1951.
11. Biggs, M. W., and A. V. Nichols. *University of California Radiation Laboratory Publication No. 2586*, 1954, p. 20.
12. Fredrickson, D. S., D. L. McCollester, R. J. Havel, and K. Ono. In *Chemistry of Lipides as Related to Atherosclerosis*, edited by I. H. Page, Springfield, Ill., Charles C Thomas, 1958, p. 205.
13. Gould, R. G. *Am. J. Med.* **11:** 209, 1951.
14. Sperry, W. M. *J. Biol. Chem.* **68:** 357, 1926.
15. Jenke, M. *Arch. exptl. Pathol. Pharmakol., Naunyn-Schmiedeberg's* **163:** 175, 1932.
16. Gould, R. G. *Proceedings of the Annual Meeting of the Council for High Blood Pressure Research, American Heart Association*, December, 1952, p. 3.
17. Biggs, M. W. *Advances in Biol. and Med. Phys.* **5:** 357, 1957.
18. Hellman, L., R. S. Rosenfeld, M. L. Eidinoff, D. K. Fukushima, T. F. Gallagher, C. I. Wang, and D. Adlersberg. *J. Clin. Invest.* **34:** 48, 1955.
19. Rosenfeld, R. S., and L. Hellman. *J. Clin. Invest.* **38:** 1334, 1959.
20. Stanley, M. M., E. P. Pineda and S. H. Cheng. *New Eng. J. Med.* **261:** 368, 1959.
21. Morris, M. D., and I. L. Chaikoff. *J. Biol. Chem.* **234:** 1095, 1959.

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The effect of biliary drainage upon the synthesis of cholesterol in the liver*

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SUMMARY

Synthesis of cholesterol and fatty acids was measured *in vitro* in the livers of rats from which the bile had been drained for various periods of time, and in control rats with normal enterohepatic circulation. Cholic and chenodeoxycholic acids were excreted at an initial rate of more than 5 mg per hour. The rate fell rapidly to a minimum, followed by a secondary rise beginning between the twentieth and thirtieth hour after cannulation. After draining the bile for 12 hours or longer, there was an increase in the synthesis of cholesterol from acetate, but not from mevalonate, and a slight depression of fatty acid synthesis. Liver cholesterol content was unchanged. Although these data suggest that the increased production of bile acids may be secondary to an increase in cholesterol synthesis, other explanations cannot be excluded.

When the common bile duct of a rat is drained continuously, the rate of excretion of bile acids decreases during the first day (1) and then increases (2), reaching a plateau by about the third day after cannulation. Thompson and Vars (2) assumed that the early fall in the rate of excretion was due to temporary damage to the liver caused by the operation, and that the plateau value indicated the normal rate of bile acid formation in the intact animal. Eriksson (3), however, has put forward evidence that the normal rate is represented by the minimum value observed toward the end of the first day, and that the plateau value represents an abnormally high rate of formation, brought about by the continual removal of bile acids through the cannula.

All the bile acids are derived from cholesterol. Moreover, oxidation to bile acids is the most important pathway for the elimination of cholesterol in rats (4). A marked increase in bile-acid formation, such as that supposed by Eriksson to occur after cannulation of the bile duct, should therefore be accompanied by an increase in the synthesis of cholesterol, provided that there is no corresponding decrease in the cholesterol content of the liver.

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In this paper we describe experiments designed to test whether there is an increase in the synthesis of cholesterol in rat liver when the bile duct is cannulated. Another object of this work was to study the time relationship between changes in bile acid excretion and cholesterol synthesis under these conditions.

METHODS

Two male albino rats of the Wistar strain (Carworth Farms), paired by weight (about 300 g), were used for each experiment. In one of each pair, the bile duct was exposed under ether anesthesia and cannulated with a fine polyethylene tube. To prevent the cannula from being pulled out, the outer end of the tube was inserted between the muscle and skin of the abdominal wall and brought to the outside through an incision in the skin of the back. After closure of the muscle and skin in separate layers, the rat was placed in a restraining cage of wire netting. Bile was collected continuously over $2^1/_2$ hour intervals in centrifuge tubes arranged in an automatic fraction collector, each tube containing 2 ml of ethanol to sterilize the bile. The other rat, used as a control, was anesthetized and the bile duct was exposed but not cannulated. After closure of the abdominal wound, it was placed in a restraining cage for the same length of time as the rat whose bile duct was

cannulated. At the end of the period of bile collection, both rats were bled from the heart and then killed by a blow on the head. The livers were removed immediately and put into ice-cold buffer solution. All the animals were given food (Rockland Rat Diet Pellets) and water ad libitum throughout the experiment. Both groups of animals lost about 5% of their total body weight per day during the experimental period. The weight losses in the control and treated animals did not differ significantly.

Preparation and Subsequent Treatment of Slices. Slices were cut by hand with a Stadie-Riggs tissue slicer (5) and were incubated in duplicate for 3 hours in 10 ml of buffer solution (6) at 37°. The incubation flask contained either sodium acetate-1-C^{14} (40 μmoles + 3 to 5 μc/g tissue) or mevalonic acid-2-C^{14} (1.5 to 4.0 μmoles + 0.10 to 0.15 μc/g tissue). At the end of the incubation, 10 ml of ethanol and 1 ml of 33% KOH were added to each flask. The flasks were then left overnight on a water bath. Cholesterol was extracted from the hydrolyzate and precipitated as the digitonide, essentially according to the method of Gould *et al.* (7). After extraction of cholesterol, the hydrolyzate was acidified with 40% H_2SO_4 and the fatty acids were extracted into redistilled light petroleum ether. The petroleum ether was washed once with 0.1% acetic acid and twice with water, and then evaporated to dryness.

Measurement of Radioactivity. In some experiments radioactivity was measured with a mica-window Geiger counter (EHM 2/S, G.E.C. Research Laboratories). The precipitates of digitonide were prepared for assay by filtering them onto perforated polyethylene planchets. The fatty acids were dissolved in 3 ml of redistilled chloroform and neutralized with 0.2 N ethanolic NaOH, using phenolphthalein as indicator. The soaps were then evaporated to dryness, dissolved in 0.5 ml of water, and transferred to planchets. The water was evaporated before measurement of the radioactivity. The yield of cholesterol digitonide or total soap was found by weighing the planchet before and after plating out the sample. The results were expressed in microcurie per gram by correcting the observed counting rate to that of an "infinitely thick" sample by means of a standard self-absorption curve, and comparing this with the counting rate given by an "infinitely thick" disk of poly(methyl-C^{14})methacrylate of known specific activity. In other experiments the precipitates of digitonide were washed and dried and then dissolved in the dioxane: phosphoric acid: absolute ethanol reagent of Kabara (8). Aliquots of these were added to a counting vial and 13 ml of the phosphor solution [0.4% phenylbiphenyloxadiazole-1,3,4(PBD) and 0.01% 1,4 bis-2(phenyloxazole)-benzene (POPOP) in toluene] was added and the sample counted in a Packard Tri-Carb liquid scintillation spectrometer. Cholesterol was determined in another aliquot using a modified Liebermann-Burchard reagent. The results were expressed in microcurie per gram by referring the observed counting rates to that given by a standard sample of known activity. The fatty acids were dissolved in 100 ml of heptane and 1-ml portions were taken for measurement of titratable acidity by the procedure recommended by Gordon (9). Five-ml samples of the heptane solution were mixed with 10 ml of the liquid phosphor in counting vials and their radioactivity was measured. The same volumes of heptane and liquid phosphor were used for measuring the background counting rate. In order to convert the results from microcurie per milliliter titratable acidity to microcurie per gram fatty acids, it was assumed that 1 equivalent of fatty acids $\cong$ 280 g.

Estimation of Bile Acids. The tubes containing the bile samples were centrifuged to remove sediment and the supernatant was decanted into 25-ml Erlenmeyer flasks. The alcohol was evaporated by heating the flasks on a steam bath for 2 hours. The procedure of Mosbach *et al.* (10) was followed for hydrolysis, extraction, and spectrophotometric estimation of cholic and chenodeoxycholic acids. When known amounts of cholic and chenodeoxycholic acids were taken through the whole procedure, including hydrolysis and extraction into ether, about 80% of each acid was recovered.

The total cholesterol in liver and plasma was determined in duplicate samples by the method of Abell *et al.* (11). In a few cases total cholesterol in the liver was determined from the cholesterol digitonide prepared during the assay for radioactivity (8).

RESULTS

Excretion of Bile and Bile Acids. The initial rate of flow of bile, estimated from the first sample obtained after insertion of the cannula, was about 1.0 ml/hour. During the first few hours the rate fell to about 0.5 ml/hour and then remained more or less constant as long as the cannula remained in position (Fig. 1).

The initial rate of excretion of cholic acid averaged 5.1 ± 0.44 mg/hour, decreasing rapidly during the first few hours to a minimum of 0.34 ± 0.08 mg/hour. The fall was usually followed by a secondary rise beginning some time between the twentieth and thirtieth hour after cannulation. During this period the rate rose to four or five times the minimum rate, but never reached values as high as those observed immediately after insertion of the cannula (Fig. 1).

The initial rate of excretion of chenodeoxycholic acid was 1.1 ± 0.08 mg/hour. Thereafter the rate followed a curve similar to that of cholic acid, but at a correspondingly lower level. The rate at the lowest part of the curve averaged 0.12 ± 0.03 mg/hour.

In two out of nine experiments continued for more than 20 hours there was no secondary rise in the rate of excretion of bile acids, although the rate of flow of bile continued at the normal rate.

Synthesis of Cholesterol. At intervals varying from 3 to 50 hours after cannulation of the bile duct, the treated rat and its control were killed, and the rate of synthesis of cholesterol from acetate and mevalonate compared in liver slices from the two animals (Table 1). The result of each experiment was expressed as the ratio of the specific activity in the cholesterol from the treated rat to that from the control rat (Fig. 2).

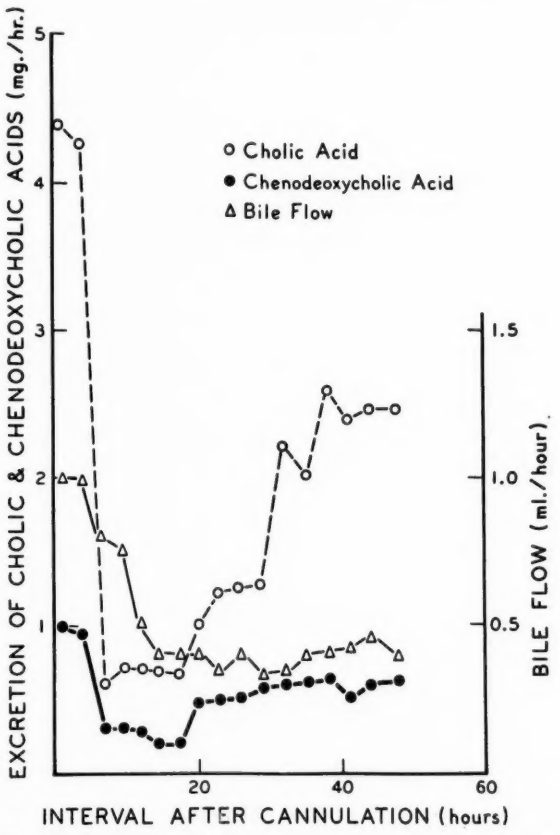

FIG. 1. Rate of flow of bile and rates of excretion of cholic and chenodeoxycholic acids from bile fistula of rat. Size of cholic acid pool in this rat estimated as follows: 25.8 mg cholic acid was excreted during 8-hour fall to minimum rate (0.7 mg/hour). If minimum rate assumed equal to rate of new formation during first 8 hours, 5.6 mg of the 25.8 mg came from new formation, therefore 20.2 mg came from preformed pool.

TABLE 1. *In Vitro* SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS BY LIVERS OF TREATED (T) AND CONTROL (C) ANIMALS AT VARIOUS INTERVALS AFTER CANNULATION OF THE BILE DUCTS

	Interval	Cholesterol		Fatty Acids
		From Acetate	From Mevalonic Acid	From Acetate
	hours	*μc/g*	*μc/g*	*μc/g*
T	3	0.51	2.37	0.186
C		1.08	3.31	0.407
T	4	1.09	5.65	0.096
C		1.76	7.24	0.164
T	4	0.87	4.99	0.057
C		1.30	6.66	0.225
T	6	0.60	1.84	
C		0.98	2.79	
T	6	0.34	5.30	0.129
C		0.46	4.20	0.072
T	8	2.52	3.47	
C		0.98	2.62	
T	12	0.65	5.64	0.169
C		0.91	6.55	0.176
T	12	1.91	3.50	0.610
C		0.82	3.36	0.741
T	12	1.18	3.26	0.077
C		0.87	3.09	0.123
T	12	1.85	1.72	0.085
C		1.11	1.35	0.073
T	18	3.16	5.10	0.286
C		1.08	5.64	0.747
T	18	1.02	1.67	0.152
C		0.58	2.58	0.376
T	18	2.00	2.38	1.554
C		0.62	1.86	2.851
T	18	1.13	5.88	1.380
C		1.10	7.15	1.459
T	24	2.64	9.00	0.258
C		0.96	6.93	0.525
T	26	0.22		
C		0.16		
T	28	3.71	8.49	0.874
C		0.96	7.06	0.551
T	28	1.09	1.55	0.134
C		0.53	1.61	0.134
T	30	1.84	0.92	0.288
C		1.59	0.90	1.297
T	47	1.87	0.83	0.205
C		0.55	0.74	0.405
T	47	1.27	2.63	0.169
C		0.37	1.98	0.119
T	50	1.03	1.03	0.486
C		1.20	3.38	0.648
T	50	1.15	1.79	
C		0.31	2.77	

Results expressed as specific activity of lipid isolated at the end of incubation. Each value is the average from paired incubations.

When the period of cannulation was longer than 12 hours, the specific activity of the cholesterol derived from acetate in the treated animals was over twice that in the control animals in eight of the thirteen experiments (mean treated/control ratio 2.43 ± 0.29). After cannulation for 12 hours there was a slight increase (the mean treated/control ratio being 1.51 ± 0.39). After shorter intervals, the results were rather variable. In one experiment at 8 hours there was an increase, but in five experiments at earlier intervals there was a slight fall.

There are not enough observations to show exactly when the rise in cholesterol synthesis occurs. However, Figure 2 suggests that it probably begins by the eighteenth hour and possibly by the twelfth, that is, before the secondary rise in bile acid excretion. This point is illustrated by an experiment shown in Figure 3 and Table 2. Two rats were killed at the eighteenth hour after cannulation and before there was any rise in bile acid excretion, yet the specific activity of the cholesterol from the treated rat was more than three times that in the control slices.

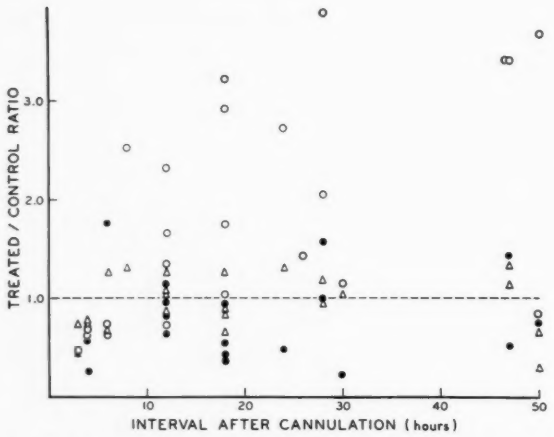

FIG. 2. Effect of cannulating the bile duct for various intervals upon the synthesis of cholesterol (○) and fatty acids (●) from acetate and of cholesterol (△) from mevalonic acid. Each value shows ratio of specific activity of cholesterol or fatty acids in treated liver to that in control liver.

When mevalonate was used as the precursor of cholesterol, the specific activity in the treated rats was, on the average, less than that in the controls, when all the results were considered together (treated/control ratio: 0.97), and when the results of experiments continued for more than 12 hours were considered separately (treated/control ratio: 0.96). In neither case was the difference between the values from treated and control rats statistically significant.

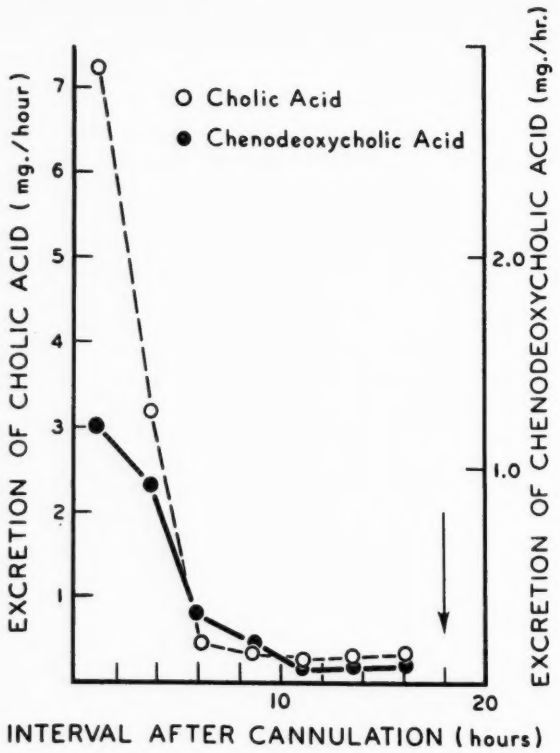

FIG. 3. Rates of excretion of cholic and chenodeoxycholic acids from rat with bile duct cannulated for 18 hours. Arrow shows time at which livers of treated and control rat removed for measurement of synthesis (see Table 2).

Synthesis of Fatty Acids. When the results of all experiments were considered together, there was a slight depression of fatty acid synthesis in the treated livers (treated/control ratio: 0.79 ± 0.10; Table 1 and Fig. 2). The difference between the values from treated and control livers was statistically significant ($0.02 < p < 0.05$).

TABLE 2. *In Vitro* SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS BY LIVERS REMOVED 18 HOURS AFTER CANNULATION OF THE BILE DUCTS

Rat	Cholesterol		Fatty Acids
	From Acetate	From Mevalonic Acid	From Acetate
	μc/g	μc/g	μc/g
Treated (T)	2 17	2.26	1.55
	1.83	2.49	
Control (C)	0.74	1.71	3 00
	0.50	2.00	2.70
T/C	3.22	1.28	0.55

Results expressed as specific activity of lipid isolated at the end of incubation.

Liver and Plasma Cholesterol. The average concentration of total cholesterol in the livers of the treated animals (186.7 ± 16.7 mg/100 g wet weight) was a little lower than that in the control livers (189.2 ± 10.4 mg/100 g wet weight), but the difference was not statistically significant. The average concentration of total cholesterol in the plasma from the treated animals (96.8 ± 8.3 mg/100 ml) was higher than that from the controls (88.5 ± 6.1 mg/100 ml). The difference was not statistically significant.

DISCUSSION

In these experiments the initial rate of excretion of cholic and chenodeoxycholic acids averaged 6.2 mg/hour. This is more than twice as high as the initial rate of excretion of bile salts observed by Eriksson (3). The difference may be due, at least in part, to the fact that in our experiments the samples were collected over shorter intervals of time. Eriksson (3) has pointed out that the first phase of excretion can hardly represent the normal rate of formation of bile acids in the intact animal, since the amount collected during the first few hours after cannulation is much greater than the amount formed during the same period, as estimated from the total exchangeable pool and the turnover time of the pool. In our rats, for instance, the initial rate of excretion of cholic and chenodeoxycholic acids is equivalent to about 150 mg/day, whereas Eriksson (12) has estimated that the amount of bile acid formed per day in the intact rat is only 2 to 3 mg/100 g rat.

It is more probable that the first samples of bile collected from the cannula come mainly from a store already present in the bile ducts, intestine, liver, and portal circulation, and that it is only when this store has been drained away that the rate of excretion truly represents the rate of formation. On this view it is possible to estimate the amount of bile acid in the preformed store, from the total collected during the early phase of excretion, less the amount newly formed during the same period, the latter being calculated on the assumption that formation occurs at a constant rate equal to the minimum observed (Fig. 1). Eriksson (3) has shown that the amount so estimated agrees roughly with the amount estimated from the extent of dilution of a labeled sample of bile acid given to a rat. This may be taken as further evidence in favor of the above interpretation of the early phase of the excretion curve. When this method of calculation was applied to our results, the amounts of cholic and chenodeoxycholic acids in the preformed store were found to average 23 ± 2.3 and 5.2 ± 1.3 mg, respectively. This agrees with the more recent results of Eriksson (12), who obtained a value of 8 mg conjugated bile acids/100 g rat.

After the initial fall in bile acid excretion, there was a second phase, lasting a few hours, during which excretion continued at a more or less constant rate, as shown in Figures 1 and 3. At this time the average rate of excretion of cholic and chenodeoxycholic acids was equivalent to 3.7 mg/day/100 g rat. This is so close to Eriksson's estimate (12) that it seems reasonable to assume that the rate of excretion observed during the second phase represents the normal rate of formation of bile acids. If this is the case, the high rates observed during the second day must reflect an increase in bile acid formation above the normal level. On this interpretation of the excretion curve, it also follows that the rise in bile acid formation does not begin until the bile duct has been drained for 20 hours or more.

The rise in cholesterol synthesis from acetate, with little or no change in synthesis from mevalonate, suggests that the increase in the treated rats is due to stimulation of a rate-limiting step between acetate and mevalonate. The existence of such a rate-limiting step in this part of the pathway from acetate to cholesterol has also been inferred by other workers who observed changes in cholesterol synthesis in the rat's liver by X-irradiation (13), injections of Triton WR-1339 (14), treatment with thyroxine (6), and cholesterol feeding (15). The observations of Bucher *et al.* (16) suggest that the rate-limiting step is the conversion of hydroxy methyl glutaryl coenzyme A to mevalonic acid. Our finding that there is a rise in cholesterol synthesis in the liver after cannulation of the bile duct is in agreement with the observations of Bergström and Gloor.[1] These workers estimated the rate of synthesis of cholesterol *in vivo* by measuring the incorporation of an intraperitoneal injection of C^{14}-acetate into the plasma cholesterol. They found an increase in cholesterol synthesis 3 to 4 days after cannulation of the bile duct.

Bergström and Danielsson (17) have shown that infusion of bile salts into the distal portion of the severed bile duct prevents the secondary rise in the bile acid excretion that occurs in a rat whose bile duct has been cannulated. They concluded that the concentration of bile acids supplied to the liver via the portal blood influences the rate of formation of bile acids in the liver and that the increase in bile acid excretion in the cannulated rat is due to release from this control. Control of the rate of formation of bile acids might be exerted upon the conversion of cholesterol to bile acids, or it might act primarily upon the synthesis of choles-

[1] Personal communication.

terol leading secondarily to an increase in the production of bile acids. Our results, suggesting that the rise in cholesterol synthesis begins before the rise in bile acid excretion, are consistent with the latter alternative. Against it, however, Gilon and Eder[2] have shown that in a cholesterol-fed rat whose bile duct was cannulated, a secondary rise in bile acid excretion occurred at about 20 hours, although cholesterol synthesis from acetate was negligible. This observation might suggest that primary control of bile acid formation is exerted upon the conversion of cholesterol to bile acids. Control at this level, however, does not explain the early rise in cholesterol synthesis in cannulated rats. Possibly the rise is due to loss of cholesterol in the bile, though this is not supported by our finding that the concentration of cholesterol in the livers of the cannulated rats is as high as that in the controls. In conclusion, our results do not exclude the possibility that control is exerted at more than one step in the formation of bile acids.

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[2] Unpublished experiments.

REFERENCES

1. Friedman, M., S. O. Byers and F. Michaelis. *Am. J. Physiol.* **164:** 786, 1951.
2. Thompson, J. C., and H. M. Vars. *Proc. Soc. Exptl. Biol. Med.* **83:** 246, 1953.
3. Eriksson, S. *Proc. Soc. Exptl. Biol. Med.* **94:** 578, 1957.
4. Siperstein, M. D., and I. L. Chaikoff. *J. Biol. Chem.* **198:** 93, 1952.
5. Stadie, W. C., and B. C. Riggs. *J. Biol. Chem.* **154:** 687, 1944.
6. Fletcher, K., and N. B. Myant. *J. Physiol.* **144:** 361, 1958.
7. Gould, R. G., C. B. Taylor, J. S. Hagerman, I. Warner, and D. J. Campbell. *J. Biol. Chem.* **201:** 519, 1953.
8. Kabara, J. J. *J. Lab. Clin. Med.* **50:** 146, 1957.
9. Gordon, R. S., Jr. *J. Clin. Invest.* **36:** 810, 1957.
10. Mosbach, E. H., H. J. Kalinsky, E. Halpern, and F. E. Kendall. *Arch. Biochem. Biophys.* **51:** 402, 1954.
11. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195:** 357, 1952.
12. Eriksson, S. *Acta Physiol. Scand.* **48:** 439, 1960.
13. Gould, R. G., and G. Popják. *Biochem. J.* **66:** 51P, 1957.
14. Bucher, N. L. R. In *Ciba Foundation Symposium on the Biosynthesis of Terpenes and Sterols*, edited by G. E. W. Wolstenholme and M. O'Connor, London, J. & A. Churchill, Ltd., 1959, p. 46.
15. Siperstein, M. D., and M. J. Guest. *J. Clin. Invest.* **39:** 642, 1960.
16. Bucher, N. L. R., P. Overath and F. Lynen. *Biochim. et Biophys. Acta* **40:** 491, 1960.
17. Bergström, S., and H. Danielsson. *Acta Physiol. Scand.* **43:** 1, 1958.

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The *in vitro* stimulation of lipogenesis and carbohydrate metabolism in liver by potassium ion and diphenylmethylacetyl-N-methylpiperazine

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SUMMARY

An experimental drug, diphenylmethylacetyl-N-methylpiperazine (A-11850), was found to stimulate the incorporation of acetate-1-C^{14}, but not that of mevalonate-2-C^{14}, into the fatty acid and nonsaponifiable fractions of rat liver slices. In addition, the drug increased about equally the oxidation of both glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}O_2$. The incubation of liver slices in a buffer containing a high level of potassium ion stimulated lipogenesis in the same manner as the drug; with potassium ion, however, the oxidation of glucose-1-C^{14} was increased to a much greater extent than that of glucose-6-C^{14}. Potassium ion also stimulated glucose oxidation in liver homogenates, whereas A-11850 did not.

Potassium ion is known to be involved in the intermediary metabolism of glucose in both plants and animals. The early work on the potassium ion requirements of certain enzymes of glucose metabolism was reviewed by Lehninger (1). Ashmore *et al.* (2) reported a stimulation of glucose metabolism by exogenous potassium ion in rat liver slices, and a similar effect was found in rat liver homogenates (3).

Potassium ion has also been observed to affect lipid metabolism. Potter (4) and Geyer *et al.* (5) demonstrated that addition of potassium ion increases the oxidation of fatty acids by liver cells and slices, and Ashmore *et al.* (6) showed that potassium increases fatty acid synthesis from glucose in liver slices. In addition, Curran and Clute (7) found that cholesterogenesis by liver cell clusters is increased by the addition of potassium. On the other hand, Kline and DeLuca (8) did not find any consistent effect of added potassium ion upon cholesterol and phospholipid synthesis from acetate in liver slices.

During an investigation of the effects of substances upon body lipids by Schmidt *et al.* (9), a group of drugs, which possesses a diarylalkylamine or related structure, was found to lower blood cholesterol levels. When these drugs were tested *in vitro*, however, they were found to enhance both lipogenesis and glucose oxidation. The effects of the drugs were then found to be similar to those of potassium ion in their stimulation of lipogenesis and glucose oxidation. The details of these studies form the subject of this paper. A portion of this work has already been presented (10).

METHODS

Livers were obtained from male Sprague-Dawley rats fed ad lib. a diet of the following composition (expressed as grams per 100 g diet): sucrose (57.0), casein (30), cottonseed oil (5.0), mineral salts (4.0), celluflour (2.0), agar (1.25), choline chloride (0.5), and vitamins (0.19). After decapitation of the rats, the livers were placed in cold saline and then either sliced with a Stadie-Riggs microtome or homogenized. The tissue preparations were then incubated in a Warburg respirometer.

For the investigation of lipogenesis in liver slices, each incubation flask contained 0.2 ml of 10% potassium hydroxide on fluted paper in the center well, 0.2 ml of the radioactive precursor in one sidearm, and 0.3 ml of 3N sulfuric acid in the other sidearm. The liver slices (200 to 250 mg) were added to 3.0 ml of buffer containing the drug or potassium ion. After gassing with 100% oxygen, the precursor was tipped in and incubation proceeded at 37°. After a specified time sulfuric acid was added and 5 minutes later the po-

tassium hydroxide in the center well was removed. The carbon dioxide was later evolved into Hyamine® for counting in a liquid scintillation counter.

The liver slices were hydrolyzed in a mixture of 11 ml of 5% sodium hydroxide in 50% ethanol for 3 hours at 75°. The nonsaponifiable fraction was obtained by extracting the hydrolyzate four times with 10 ml of hexane, and the fatty acids by a similar extraction after acidification of the hydrolyzate with hydrochloric acid. The combined extracts containing the nonsaponifiable fraction were washed once with a dilute solution of sodium acetate; the extracts containing fatty acids were washed once with very dilute acetic acid. Control experiments showed that lipids were usually completely removed from the hydrolyzate by three extractions, and that single washes of the extracts effectively removed contaminating C^{14}-acetate. It should be noted that the proportions of reagents were adjusted so that after 30 seconds of vigorous agitation on a buzzer the emulsions separated cleanly, usually within 1 to 4 minutes. Hexane solutions of the lipids were counted in a liquid scintillation counter.

Two buffers were used for the incubation of slices: a modified Krebs-Ringer phosphate buffer, pH 7.0–7.1, which contained one-half of the standard amount of calcium ion, and a high potassium ion buffer, pH 7.0–7.1, in which all the sodium salts of the preceding buffer were replaced with the corresponding potassium salts. Intermediate concentrations of potassium ion in buffers were obtained by mixing suitable proportions of these two buffers.

Liver homogenates were prepared in a sodium phosphate buffer (3.3 g liver/20 ml buffer) according to the procedure of Wenner *et al.* (3) with a Teflon-glass homogenizer. The homogenates were then incubated in high sodium ion or in high potassium ion buffers which had been reinforced with DPN[1] and cytochrome c (3). In contrast to the experiments with slices, sulfuric acid was not added at the end of the homogenate incubations because it coagulated the tissue and hindered its removal from the incubation flasks. The tissue and the medium were hydrolyzed together, and after acidification of the hydrolyzate, the total lipids were extracted with hexane. Again, the $C^{14}O_2$ was later evolved into Hyamine® for counting.

The use of a bicarbonate buffer would probably have increased lipogenesis above that of the present buffer systems. Drugs at certain levels and conditions, however, can produce a general toxic effect in slices and homogenates which is evidenced by reduced oxygen uptake and carbon dioxide formation. Trapping of carbon dioxide permits the detection of such an effect by an accurate determination of the oxygen uptake. Moreover, the experiments in the present paper were designed primarily to compare controls and treatments, and maximal lipogenesis was considered of secondary importance to a determination of the oxygen uptake.

For the experiments in which the lipids were chromatographed on silicic acid, 1 g of liver slices was incubated in 125-ml Warburg flasks with the acetate-1-C^{14} and other materials increased proportionately. After a 3-hour incubation period, the lipids were extracted from the slices with 10 ml of Delsal's reagent (11) per gram of liver. The extraction was repeated twice and the solvent combined and evaporated *in vacuo*. Lipids were extracted from the residue with several portions of warm hexane and the clear centrifuged solution was added to a 14 mm × 200 mm column of silicic acid (chromatographic silica from the G. Frederick Smith Company). The silicic acid was packed with hexane without any preliminary treatment. Lipid fractions were eluted from the column according to the procedure of Fillerup and Mead (12, 13).

Diphenylmethylacetyl-N-methylpiperazine (A-11850) was prepared by Mr. R. J. Michaels and Mr. H. B. Wright of these laboratories. The acetic acid-1-C^{14} and the C^{14}-glucoses were obtained from the New England Nuclear Company; the purity of the glucoses was confirmed by spraying and determining the radioactivity of a paper chromatogram run in butanol-acetic acid. Unlabeled mevalonic acid and the mevalonic acid-2-C^{14} were obtained from the Isotopes Specialties Company as the diphenylethylenediamine salts and these were regenerated prior to use by ether extraction of an alkaline solution. Acetoacetate was determined by the method of Edson (14) with aniline citrate, the radioactive carbon dioxide being evolved into Hyamine®. Unless otherwise noted, the radiochemical yield of an incubation was defined as the per cent of the added radioactivity recovered in a given substance per 100 mg of fresh tissue per hour. Oxygen uptakes (Q_{O_2}) are expressed either as microliter per hour per milligram of fresh tissue slice or as microliter per hour per milliliter of homogenate.

RESULTS

Effect of Graded Levels of A-11850 and Potassium Ion upon Lipogenesis in Rat Liver Slices. The influence of different levels of A-11850 and potassium ion upon the incorporation of acetate-1-C^{14} into lipids is shown in Figure 1, where it can be seen that both

[1] Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; HMG, β-hydroxy-β-methylglutaric acid; CoA, coenzyme A.

activators produced similar responses. Fatty acid synthesis in the presence of 155 mM potassium ion and 0.65 mM A-11850 was increased to 500% and 700%, respectively, of the control value in Krebs-Ringer buffer. In the same incubations the corresponding data for acetate incorporation into the nonsaponifiable fraction were 250% and 220%. Since the incubations with the drug were carried out in Krebs-Ringer phosphate, each of these incubations was also 5.6 mM in potassium ion. It should also be noted that in the subsequent experiments A-11850 was used at a level of 0.65 mM. To demonstrate stimulation by potassium ion, the previously described buffer was used which contained 155 mM potassium ion. It was not realized until many experiments had been completed that this level of potassium ion was greater than the optimum level. The results of all the incubations using such concentrations of A-11850 and potassium ion are given in Table 1, and the data clearly demonstrate the pronounced stimulation of lipogenesis by both substances. As indicated in Figure 1, the effects of potassium ion upon lipogenesis would probably have been greater at a lower level. In the potassium ion- and drug-stimulated incubations, neither the respiration nor the incorporation of acetate into carbon dioxide and acetoacetate increased above control levels. Moreover, the presence of 100 mM glucose in the medium did not affect the respiration or the acetate incorporation into lipids and carbon dioxide.

Chromatographic Separation of Lipids. Lipid extracts were prepared from incubations (1 g of rat liver slices) which were five times the size of those described in Figure 1. The slices were incubated in either the 155 mM potassium ion buffer or the Krebs-Ringer phosphate buffer containing 0.65 mM A-11850. The respiration and the incorporation of acetate-1-C^{14} into the carbon dioxide and lipids were comparable to those of the smaller incubations. When the lipid extracts were passed over silicic-acid columns, effluent

TABLE 1. STIMULATION OF LIPOGENESIS FROM ACETATE-1-C^{14} IN RAT LIVER SLICES*

	Krebs-Ringer Phosphate	A-11850 (0.65 mM)	Potassium Ion (155 mM)
QO_2	1.42 ± 0.05 (9)	1.36 ± 0.04 (7)	1.19 ± 0.02 (5)
	Radiochemical Yield (Per Cent)		
Acetoacetate	1.28 ± 0.04 (2)	1.14 ± 0.10 (2)	1.36 ± 0.04 (2)
Nonsaponifiable lipid	0.03 ± 0.00 (8)	0.11 ± 0.02 (6)	0.08 ± 0.03 (4)
Fatty acids	0.08 ± 0.01 (8)	0.47 ± 0.09 (6)	0.53 ± 0.11 (4)
Carbon dioxide	2.14 ± 0.31 (9)	2.26 ± 0.40 (7)	2.83 ± 0.43 (5)

* Incubations were effected as described in Figure 1. The number of individual livers is given in parentheses and each liver was incubated in triplicate. Each value is expressed as a mean ± the standard error of the mean.

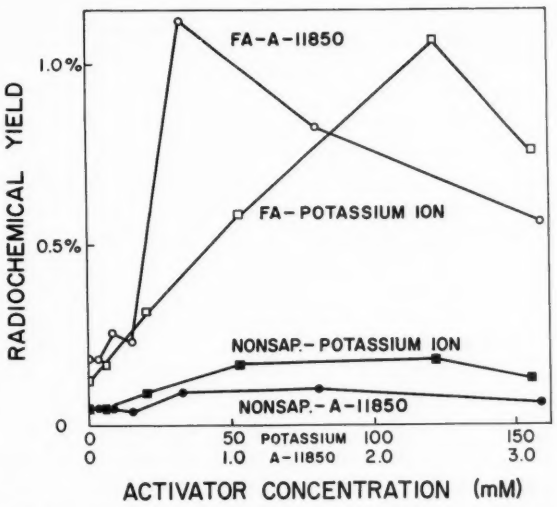

FIG. 1. Effect of graded levels of potassium ion and A-11850 upon lipogenesis by rat liver slices. Flask components: 3.0 ml modified Krebs-Ringer phosphate buffer with graded amounts of potassium ion or A-11850 and 200 to 250 mg liver slices in the main well, 0.2 ml 10% KOH in center well, 0.3 ml 3N H_2SO_4 in sidearm, and 0.2 ml of 104 mM acetate-1-C^{14} in other sidearm. Incubated for 3 hours at 37° under 100% oxygen. O = A-11850 and fatty acids; ● = A-11850 and nonsaponifiable lipid; □ = potassium ion and fatty acids; ■ = potassium ion and nonsaponifiable lipid.

curves of the radioactivity were obtained as shown in Figure 2. The peaks correspond to those originally described by Fillerup and Mead (12), i.e., hydrocarbons were eluted by hexane, cholesterol esters by 1% ether in hexane, triglycerides by 3% ether in hexane,

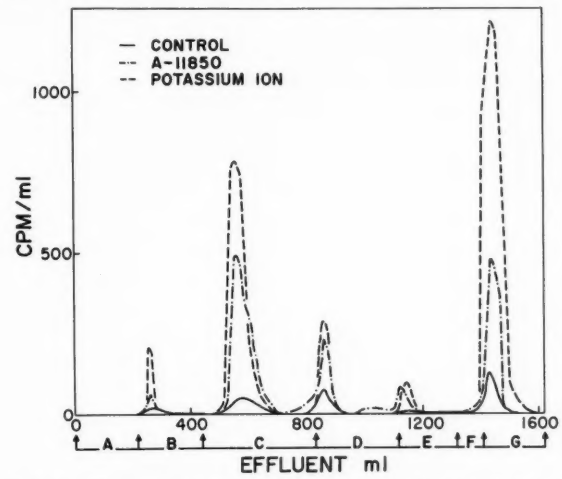

FIG. 2. Chromatography of lipids from liver slice incubations. The incubations were five times the size of those described in Figure 1; silicic-acid column size, 1.4 × 20 cm; fraction size, 24 ml. Solvent mixture *A*, hexane; *B*, 1% diethyl ether in hexane; *C*, 3% ether in hexane; *D*, 10% ether in hexane; *E*, 50% ether in hexane; *F*, 100% ether; *G*, 100% methanol.

free cholesterol by 10% ether in hexane, fatty acids by 50% ether in hexane, and finally phospholipids by both 100% ether and 100% methanol. However, the free fatty acids in the extracts and the known sample of C^{14}-fatty acids (obtained from a prior incubation) must have become oxidized during their isolation, since in a later report Mead and Fillerup (13) observed that unoxidized fatty acids are eluted directly after the triglycerides. Identification of the substances in each of the other peaks was also confirmed by chromatography with known lipids. Thus the elution patterns shown in Figure 1 demonstrate that acetate-1-C^{14}, under the influence of A-11850 and potassium ion, became incorporated into many types of lipids.

Differential Stimulation of Lipogenesis from Acetate and Mevalonate in Rat Liver Slices. The data obtained when either acetate-1-C^{14} or mevalonate-2-C^{14} was incubated in the presence of 0.65 mM A-11850 with slices from the same liver are shown in Table 2. As may be seen, the expected increase in the labeling of the nonsaponifiable fraction from acetate was obtained, but the labeling from mevalonate remained unchanged. In Table 3 are data from a comparable incubation in the high potassium ion buffer which show a similar differential incorporation into the nonsaponifiable fraction. With neither substance was there any suggestion of an increased labeling from mevalonate in these or other similar experiments.

Stimulation of the Conversion of Glucose to Carbon Dioxide and Lipids in Rat Liver Slices. Because the stimulation of lipogenesis from acetate-1-C^{14} by A-11850 and potassium ion were so similar, it was of interest to see whether this similarity also extended to glucose metabolism. The data in Table 4 show that both substances increased the oxidation of glucose-1-C^{14} and glucose-6-C^{14} to $C^{14}O_2$. It is evident from the data that the oxidation of glucose-1-C^{14} was stimulated to a considerable extent by the addition of potassium ion, while that of glucose-6-C^{14} was almost negligible. On the other hand, A-11850 significantly increased the oxidation of both glucoses, apparently increasing the oxidation of glucose-1-C^{14} slightly more than that of glucose-6-C^{14}. Thus, in contrast to their similar stimulation of lipogenesis from acetate, the effects of A-11850 and potassium ion upon glucose oxidation differ from each other. A similar stimulation of glucose oxidation by A-11850 was also observed when incubations were carried out in a Krebs-Ringer bicarbonate buffer instead of the usual Krebs-Ringer phosphate buffer.

It may be seen in Table 5 that the addition of either A-11850 or potassium ion also increased the incorporation of the C^{14}-glucoses into the nonsaponifiable material and fatty acids. A-11850 increased the incorporation of glucose-6-C^{14} slightly more, on an absolute basis, than that of glucose-1-C^{14}. However, after the addition of potassium ion to liver slices, there was a much greater incorporation into lipids of glucose-6-C^{14} than of glucose-1-C^{14}. It should also be noted that whereas both A-11850 and potassium ion caused larger increases in the amount of lipogenesis from glucose-6-C^{14} than from glucose-1-C^{14}, the converse was observed for the oxidations to $C^{14}O_2$, as is demonstrated in Table 4.

The incorporation of C^{14}-glucose into lipids by liver slices was extremely low even though the oxidation of glucose to $C^{14}O_2$ and lipogenesis from C^{14}-acetate occurred at rates comparable to those observed by other investigators. In fact, of the 10 rats used in Table 4, slices from only 5 livers incorporated enough C^{14}-glucose into fatty acids and the nonsaponifiable fraction to give significant radioactivity.

TABLE 2. DIFFERENTIAL STIMULATION OF LIPOGENESIS FROM ACETATE AND MEVALONATE IN LIVER SLICES BY A-11850*

Additions	Radiochemical Yield (Per Cent)		
	Nonsaponifiable Lipid	Fatty Acids	Carbon Dioxide
	Precursor: Acetate-1-C^{14}		
None	0.03 ± 0.01	0.02 ± 0.01	0.96 ± 0.03
A-11850	0.20 ± 0.02	0.17 ± 0.01	1.10 ± 0.02
	Precursor: Mevalonate-2-C^{14}		
None	0.90 ± 0.07	0.01 ± 0.01	0.11 ± 0.02
A-11850	0.76 ± 0.01	0.01 ± 0.01	0.09 ± 0.01

* Acetate-1-C^{14}, mevalonate-2-C^{14}, and A-11850 were at 0.65 mM concentrations. Liver slices were incubated in the modified Krebs-Ringer phosphate for 3 hours at 37° under 100% oxygen. Recoveries are expressed as radiochemical yields and each value is the mean of triplicate incubations. All the data were obtained with one rat liver.

TABLE 3. DIFFERENTIAL STIMULATION OF LIPOGENESIS FROM ACETATE AND MEVALONATE IN LIVER SLICES BY POTASSIUM ION*

Medium	Radiochemical Yield (Per Cent)		
	Nonsaponifiable Lipid	Fatty Acids	Carbon Dioxide
	Precursor: Acetate-1-C^{14}		
KRP-buffer	0.04 ± 0.00	0.13 ± 0.00	1.83 ± 0.17
Potassium ion buffer (155 mM)	0.16 ± 0.00	0.75 ± 0.16	1.99 ± 0.00
	Precursor: Mevalonate-2-C^{14}		
KRP-buffer	1.09 ± 0.04	0.03 ± 0.00	0.14 ± 0.03
Potassium ion buffer (155 mM)	1.04 ± 0.09	0.04 ± 0.01	0.14 ± 0.02

* Incubations were carried out as described for those in Table 2.

TABLE 4. STIMULATION OF GLUCOSE OXIDATION IN LIVER SLICES*

Rat	Radiochemical Yield of CO_2 (Per Cent)	
	G-1-C^{14}	G-6-C^{14}
	Krebs-Ringer Phosphate	
3	0.32 ± 0 02	0.10 ± 0.01
4	0.17 ± 0.01	0.05 ± 0 01
5	0.23 ± 0.02	0.06 ± 0.00
6	0.20 ± 0.02	0.07 ± 0.01
7	0.09 ± 0.01	0.08 ± 0.01
8	0.19 ± 0.01	0.08 ± 0.00
9	0.19 ± 0.00	0 08 ± 0 00
10	0.18 ± 0.01	0.07 ± 0.00
11	0.24 ± 0.01	0.09 ± 0.01
12	0.19 ± 0.01	0.07 ± 0.00
	A-11850 (0.65 mM)	
3	0.56 ± 0.02 (0.24)	0.23 ± 0.04 (0.13)
4	0.32 ± 0.05 (0.15)	0.12 ± 0.01 (0.07)
6	0.41 ± 0.02 (0.21)	0 15 ± 0.01 (0.08)
10	0.40 ± 0.01 (0.22)	0.12 ± 0.02 (0.05)
11	0.37 ± 0.02 (0.13)	0.23 ± 0.02 (0.14)
12	0.32 ± 0.00 (0.13)	0.15 ± 0.01 (0.08)
	($\bar{X}$ = 0.18 ± 0.02)	($\bar{X}$ = 0.09 ± 0.02)
	High Potassium Ion Buffer (155 mM)	
3	1.23 ± 0.26 (0.91)	0 14 ± 0.01 (0.04)
5	0.86 ± 0.04 (0.63)	0.13 ± 0.01 (0.07)
7	0.29 ± 0.03 (0.20)	0.13 ± 0.00 (0.05)
8	0.36 ± 0.04 (0.17)	0.09 ± 0.00 (0 01)
9	0.33 ± 0.04 (0.14)	0.08 ± 0.01 (0.00)
	($\bar{X}$ = 0 41 ± 0.17)	($\bar{X}$ = 0.03 ± 0.01)

* Glucose was added to the medium at a level of 0.4 mM. The incubations proceeded for 90 minutes at 37° under 100% oxygen. Each yield is a mean of triplicate incubations and is expressed as a mean ± standard error of the mean. Values in parentheses are the increases in glucose oxidation after the addition of either A-11850 or potassium ion.

Actions of A-11850 and Potassium Ion upon Acetate and Glucose Metabolism of Rat Liver Homogenates. In order to determine whether A-11850 and potassium ion would stimulate metabolism in a cell-free system, experiments using homogenates were compared with those of slices (Table 6). As reported earlier by Wenner *et al.* (3), the addition of potassium ion was found to increase both the respiration and glucose oxidation of liver homogenates reinforced with cytochrome c and DPN. On the other hand, 0.65 mM A-11850 depressed respiration but not glucose oxidation. In two separate experiments the oxidation of acetate-1-C^{14} to $C^{14}O_2$ was also found to be increased by potassium ion but not by A-11850; lipogenesis was slightly inhibited in both experiments. These actions are in contrast to those observed in slices where both A-11850 and potassium ion caused stimulations.

TABLE 5. STIMULATION OF C^{14}-GLUCOSE CONVERSION TO LIPIDS IN LIVER SLICES

Rat	Radiochemical Yield (Per Cent)*	
	Glucose-1-C^{14}	Glucose-6-C^{14}
6	Krebs-Ringer Phosphate	
Nonsaponifiable lipid	0.19 ± 0.03	0.42 ± 0.04
Fatty acids	0.74 ± 0.15	1.3 ± 0.1
	A-11850 (0.65 mM)	
Nonsaponifiable lipid	1.4 ± 0.2 (1.2)	3.1 ± 0.4 (2.7)
Fatty acids	7.6 ± 0.9 (6.9)	15.0 ± 1.3 (13.7)
12	Krebs-Ringer Phosphate	
Nonsaponifiable lipid	0.19 ± 0.05	0.41 ± 0.05
Fatty acids	0.56 ± 0.06	1.1 ± 0.1
	A-11850 (0.65 mM)	
Nonsaponifiable lipid	0.73 ± 0.06 (0.5)	1.9 ± 0.5 (1.5)
Fatty acids	3.1 ± 0.2 (2.5)	7.4 ± 1.3 (6.3)
7	Krebs-Ringer Phosphate	
Nonsaponifiable lipid	0.00 ± 0.03	0.12 ± 0.03
Fatty acids	0.19 ± 0.03	0.67 ± 0.05
	High Potassium Ion Buffer (155 mM)	
Nonsaponifiable lipid	0.00 ± 0.04 (0.00)	0.72 ± 0.06 (0.6)
Fatty acids	0.33 ± 0.01 (0.14)	2.8 ± 0.6 (2.1)
9	Krebs-Ringer Phosphate	
Fatty acids	0.26 ± 0.05	0.52 ± 0.09
	High Potassium Ion Buffer (155 mM)	
Fatty acids	0.32 ± 0.02 (0.04)	0.77 ± 0.08 (0.25)

* Actual yields × 10^3. Triplicate incubations were carried out as described in Table 4. Values in parentheses are the increases in lipogenesis after the addition of either A-11850 or potassium ion.

TABLE 6. STIMULATION OF GLUCOSE OXIDATION IN LIVER HOMOGENATES*

Oxygen Uptake ($\mu l/hr/ml$)	Radiochemical Yield of Carbon Dioxide (Per Cent)	
	Glucose-1-C^{14}	Glucose-6-C^{14}
High Sodium Ion Buffer		
221 ± 14	0.30 ± 0.00	0.008 ± 0.001
A-11850 (0.65 mM) in Sodium Ion Buffer		
143 ± 5	0.31 ± 0.02	0.007 ± 0 000
Potassium Ion Buffer (88 mM)		
284 ± 11	0.31 ± 0.01	0.029 ± 0.001

* The homogenates were incubated in air for 1 hour at 37°. C^{14}-glucose was present at 10 mM. The $C^{14}O_2$ yields and the oxygen uptakes are expressed as the means ± the standard error of the mean for duplicate and quadruplicate incubations, respectively.

DISCUSSION

The dependence of lipogenesis upon glucose metabolism has been investigated and reviewed by Siperstein and Fagan (15). They presented evidence that in liver homogenates the effects of glucose metabolism are mediated through the formation of reduced pyridine nucleotides. In addition, Ashmore *et al.* (6) have shown that both glucose metabolism and fatty acid synthesis are elevated by potassium ion. The conclusion is that lipogenesis can be regulated by glucose catabolism and that this in turn may be influenced by potassium ion. In the *in vitro* systems used in this paper, which are similar to those used by many other investigators, a synthetic drug as well as potassium ion exerted such a role. The increases in lipogenesis and oxidation of glucose in the presence of either A-11850 or potassium ion are further examples of an increased glucose oxidation being accompanied by an increased lipogenesis; glucose oxidation and lipogenesis are known to be increased by the addition of DPN and TPN to liver homogenates (15) and also by the addition of insulin to adipose tissue (16).

The data in Tables 2 and 3 demonstrate that in liver slices both the drug and potassium ion increase labeling of the nonsaponifiable fraction from acetate-1-C^{14} but not from mevalonate-2-C^{14}. Furthermore, as shown in Table 1, the radioactivity of acetoacetate in the medium after incubation with labeled acetate was found to be the same in both control and stimulated incubations. (In two separate experiments the absolute amounts of acetoacetate in the medium were also found to be the same.) In contrast to the earlier hypothesis that acetoacetic acid is formed by deacylation of acetoacetyl-CoA, Lynen *et al.* (17) reported that liver acetoacetate is derived mainly from the cleavage of HMG-CoA, and therefore its specific activity should reflect that of HMG-CoA. A consideration of the above observations suggests that A-11850 and potassium ion do not affect the specific activity of HMG-CoA, but that they stimulate some process(es) in the conversion of HMG-CoA to mevalonate. The evidence presented here, together with that of other investigators, suggests that this stimulatory effect upon the conversion is mediated through an increased oxidation of glucose and its concomitant formation of reduced TPN which is known to be the coenzyme involved in the conversion of HMG-CoA to mevalonic acid (18).

Although the actions of A-11850 and potassium ion upon lipogenesis from acetate-1-C^{14} and mevalonate-2-C^{14} were found to be very similar, differences were observed in their stimulation of glucose metabolism. Examination of the data in Tables 4 and 5 shows that although each of the substances increased the conversion of glucose-1-C^{14} and glucose-6-C^{14} to carbon dioxide and lipids, potassium caused a proportionately greater increase in the oxidation of glucose-1-C^{14} than of glucose-6-C^{14}, and also a proportionately greater stimulation of lipogenesis from glucose-6-C^{14} than from glucose-1-C^{14}. These data suggest that there are changes in the relative quantities of glucose passing through Embden-Meyerhoff glycolysis and the hexose phosphate shunt. However, in view of the discussion by Katz and Wood (19), any quantification based only upon glucose-1-C^{14} and glucose-6-C^{14} would be equivocal.

Potassium ion will not increase glucose oxidation in some types of homogenates; no activation was observed in two trials with homogenates prepared according to Dituri *et al.* (20) with added nicotinamide. However, when plain liver homogenates were reinforced with cytochrome c and DPN, an increased glucose oxidation was found in the presence of potassium ion (Table 6), as Wenner *et al.* (3) reported earlier. The elevated oxidation of acetate after addition of potassium ion to the homogenate may be due to its effect upon the acetate activating enzyme as Von Korff (21) observed in heart extracts.

Although both substances increased metabolism in a cellular system (liver slices), only potassium ion stimulated metabolism in liver homogenates. It may be that A-11850 is involved with intracellular potassium ion, although we have no evidence for it; A-11850, however, is structurally related to many antihistaminic drugs, and Judah (22) has reported that such drugs enable liver slices to accumulate potassium ion. It is also of interest that antihistaminics are known to lower serum potassium ion levels in rats (23) and to produce hypoglycemia in man and animals (24, 25). In this connection it should be noted that Hastings *et al.* (26) discussed the possibility of such a cellular relationship between insulin and potassium ion, and that insulin has been found to increase the potassium content of rat diaphragm during incubation *in vitro* (27) and to reduce hyperkalemia (28). These observations emphasize certain relationships among insulin, potassium ion, and drugs having antihistaminic activity.

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REFERENCES

1. Lehninger, A. L. *Physiol. Revs.* **30**: 393, 1950.
2. Ashmore, J., G. F. Cahill, Jr., A. B. Hastings, and S. Zottu. *J. Biol. Chem.* **224**: 225, 1957.
3. Wenner, C. E., D. F. Dunn and S. Weinhouse. *J. Biol. Chem.* **205**: 409, 1953.
4. Potter, V. R. *J. Biol. Chem.* **163**: 437, 1946.
5. Geyer, R. P., M. F. Meadows, L. D. Marshall, and M. S. Gongaware. *J. Biol. Chem.* **205**: 81, 1953.
6. Ashmore, J., G. Weber and B. R. Landau. *Cancer Research* **18**: 974, 1958.
7. Curran, G. L., and O. L. Clute. *J. Biol. Chem.* **204**: 215, 1952.
8. Kline, D. L., and H. A. DeLuca. *Can. J. Biochem. and Physiol.* **34**: 429, 1956.
9. Schmidt, J. L., D. L. Martin and G. H. Barlow. American Society of Pharmacology and Experimental Therapy, Fall Meeting, Ann Arbor, Mich., 1958.
10. Minard, F. N. *Proc. Can. Federation Biol. Socs.* **2**: 47. 1959.
11. Delsal, J. L. *Bull. soc. chim. biol.* **26**: 99, 1944.
12. Fillerup, D. L., and J. F. Mead. *Proc. Soc. Exptl. Biol. Med.* **83**: 574, 1953.
13. Mead, J. F., and D. L. Fillerup. *Proc. Soc. Exptl. Biol. Med.* **86**: 449, 1954.
14. Edson, N. L. *Biochem. J.* **29**: 2082, 1935.
15. Siperstein, M. D., and V. M. Fagan. *J. Clin. Invest.* **37**: 1185, 1958.
16. Winegrad, A. I., and A. E. Renold. *J. Biol. Chem.* **233**: 273, 1958.
17. Lynen, F., U. Henning, C. Bublitz, B. Sorbo, and L. Kroplin-Rueff. *Biochem. Z.* **330**: 269, 1958.
18. Durr, I. F., and H. Rudney. *J. Biol. Chem.* **235**: 2572, 1960.
19. Katz, J., and H. G. Wood. *J. Biol. Chem.* **235**: 2165, 1960.
20. Dituri, F., W. N. Shaw, J. V. B. Warms, and S. Gurin. *J. Biol. Chem.* **226**: 407, 1957.
21. Von Korff, R. W. *J. Biol. Chem.* **203**: 265, 1953.
22. Judah, J. D. *Nature* **187**: 506, 1960.
23. Halpern, B. N., M. Briot, D. Mouton, and J. Truffert. *Compt. rend. soc. biol.* **149**: 1223, 1955.
24. Goldner, M. G. *A.M.A. Arch. Internal Med.* **102**: 830, 1958.
25. Zwang, H., and M. Jacubeit. *Biochem. Z.* **323**: 355, 1952.
26. Hastings, A. B., A. E. Renold and Ching-Tseng Teng. In *Recent Progress in Hormone Research*, edited by G. Pincus, New York, Academic Press, Inc., 1955, vol. 11, p. 381.
27. Ungar, G., and S. Kadis. *Nature* **183**: 49, 1959.
28. Bywaters, E. G. L. *J. Am. Med. Assoc.* **124**: 1103, 1944.

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Effects of medium fatty acid concentration, epinephrine, and glucose on palmitate-1-C^{14} oxidation and incorporation into neutral lipids by skeletal muscle *in vitro*

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SUMMARY

The rate of oxidation of palmitate-1-C^{14} to $C^{14}O_2$ by rat skeletal muscle *in vitro* was shown to increase markedly as a function of the concentrations of free fatty acids (FFA) in the medium. Neither epinephrine nor glucose had any important effect. The rate of incorporation of palmitate-1-C^{14} into tissue neutral lipids was also increased by higher levels of FFA in the medium. At any given concentration of FFA this incorporation was also increased by glucose but was not altered by epinephrine. It is concluded that the deposition of triglyceride reported to occur in muscle after epinephrine or norepinephrine administration is due to the effects of these hormones on serum FFA levels and not to a direct effect on muscle metabolism. The profound effect of FFA concentration on the rate of FFA oxidation suggests that regulation of the serum concentrations of this labile lipid fraction may be of central importance in determining over-all rates of body metabolism. In particular, it is proposed that the elevated metabolic rates after epinephrine administration and in hyperthyroidism may, in part, be directly attributable to the elevations of serum FFA in these conditions.

Evidence accumulating over the past several years, both from studies *in vivo* and *in vitro*, indicates the important role of lipids in muscle metabolism. From studies of arteriovenous differences across the human forearm, Andres *et al.* (1) showed that the major substrate could not be carbohydrate and, from the fact that the respiratory quotient found was 0.80, concluded that oxidation of lipid must account for the larger part of the metabolic activity. Over short time intervals the muscle can apparently utilize its endogenous supplies of lipid (2, 3), but over any extended period of time it must ultimately depend upon substrate transported from adipose tissue depots. The work of Gordon and Cherkes (4) and of Dole (5) led to the elucidation of the central role of the serum free (or unesterified) fatty acids (FFA) in the transport of lipid from depots to sites of utilization. Direct studies of arteriovenous differences have established this lipid fraction as a primary source of fat calories in cardiac muscle, and probably also in skeletal muscle (6).

The question then arises as to the site at which homeostatic control of the rate of lipid oxidation is exercised. Since the muscle mass probably accounts for the largest fraction of the fat calories utilized as an energy source, the question of the mechanism of control is especially pertinent in that tissue. Is control of the amount of fat oxidized exercised predominantly within the muscle tissue, for example, by hormonal regulation of metabolic rates or by substrate-concentration feedback? Or is the control exercised by adjustments in the concentration of extracellular FFA made available to the tissue? Both general types of control may, of course, be involved. One purpose of the present studies was to test the relative importance of FFA concentration in determining the rate of its oxidation by muscle.

A second purpose was to explore the relation between FFA concentration and the rate of triglyceride synthesis. Studies by Feigelson *et al.* (7) in this laboratory have shown that when serum FFA concentration is maintained at a high level by constant intravenous

infusion of epinephrine or norepinephrine, there is a rapid and marked increase in liver triglyceride concentration and, although to a lesser extent, in skeletal muscle triglyceride concentration. Maling and Highman (8) have observed deposition of neutral fat in the myocardium after intravenous infusion of large doses of either epinephrine or norepinephrine. It was of interest to determine whether triglyceride deposition induced by catecholamines might be due to some direct effect on muscle metabolism or whether it could be adequately explained on the basis of the elevated serum FFA concentration secondary to stimulation of FFA release from adipose tissue (4, 5, 9).

Finally, in view of the inverse relationship between the relative rates of lipid oxidation and the availability of glucose in the intact animal, as indicated by changes in respiratory quotient, it was of interest to determine whether glucose would, in an isolated system, influence the rate of oxidation of FFA. The alternative possibilities are, again, that control is exercised exclusively by adjustments of the rate of release of FFA from adipose tissue, which has been shown to occur (6), or that both mechanisms operate.

METHODS AND MATERIALS

Male Sprague-Dawley rats fed ad libitum on a standard laboratory chow diet were used. An extensor muscle lying beneath the gastrocnemius muscle was dissected free *in toto*. The paired muscle from each rat was used as a control. These muscles weighed approximately 50 mg and measured approximately 13 × 1 × 3 mm.

The tissues were incubated in 3 ml of Krebs-Ringer phosphate buffer (10), pH 7.4, containing 3% bovine serum albumin (Armour Pharmaceutical Company, list 2293, fraction V from bovine plasma). The Q_{O_2} of these muscle strips measured in the absence of added substrate was 4.1, 4.2, and 5.2 μl/mg (dry)/hour in preparations from three different rats. Fritz *et al.* (11) have reported Q_{O_2} values of 5.79 for diaphragm, and 2.34 for *L. dorsi* muscle.

The unlabeled palmitic acid was Fisher Reagent Grade, and the palmitic acid-1-C^{14} was obtained from Nuclear Chicago Corporation, Desplaines, Illinois. The unlabeled palmitic acid was dissolved in hot 0.154 M NaCl by neutralization with equivalent amounts of 2 N KOH and was then added to the appropriately concentrated medium. Twenty-five microliters of palmitic acid-1-C^{14}, representing approximately 25,000 cpm/ml of final medium, was added to each incubation flask.

The basic albumin-containing medium contained 0.23 μeq/ml of titratable FFA, which was 53.5% palmitic acid, as shown by gas-liquid chromatography. The unlabeled palmitic acid added to raise the medium FFA concentration was found actually to contain only 85% palmitic acid. These values were used to calculate the true dilution of precursor palmitate-1-C^{14} when unlabeled palmitate was added at different concentrations to the basic medium. In the results reported below, the amount of palmitate metabolized is, in every case, expressed as the minimum number of μeq of medium palmitate oxidized or incorporated, based on the total recovered radioactivity in each fraction studied, divided by the calculated specific radioactivity of the medium palmitate.

The addition of internal standards showed that the added unlabeled palmitic acid did not exert a quenching effect in the determination of the radioactivity of the medium. It was also shown that the specific activity of the medium palmitic acid-1-C^{14} was not affected by the order of addition of labeled and unlabeled palmitic acid.

In the experiments using epinephrine, the final concentration used was 3 μg/ml. The glucose concentrations were determined enzymatically, using "Glucostat" reagents obtained from the Worthington Biochemical Corporation, Freehold, New Jersey.

Incubation Procedure. The rats were killed by swift decapitation, and the paired muscles were immediately dissected out, weighed, and dropped into 3 ml of medium in 25-ml Erlenmeyer flasks. The flasks were then flushed with oxygen for 5 minutes in an incubator at 38°, shaking at 60 cycles per minute. The flasks were then recapped with a rubber plug containing a glass cup holding 0.3 cc of 30% KOH and a 2 × 1 cm piece of folded Whatman #2 filter paper. At the end of a 2-hour incubation, 0.3 cc of 4 N H_2SO_4 was injected, and the flasks were shaken for an additional 20 minutes to ensure complete CO_2 collection. The contents of the glass cup, including the filter paper, were then washed into a graduated cylinder with distilled water to a final volume of 10 cc. After the addition of 0.1 cc of 1:10 Triton GR-5 detergent (Rohm and Haas, Inc., Philadelphia, Pa.), 3 ml of the aqueous solution containing collected $C^{14}O_2$ was pipetted onto 1 g of anthracene crystals (Distillation Products Industries, Rochester, N.Y.; blue-violet fluorescent grade) in counting vials. The $C^{14}O_2$ was then counted in a Packard Tri-carb liquid scintillation spectrometer according to the method of Steinberg (12).

The muscle was removed at the end of the incubation, rinsed twice in distilled water, blotted, and homogenized in 3 ml of distilled water. The homogenate was washed into a 60-ml separatory funnel with three

10-ml aliquots of the extraction mixture described by Dole (5), modified only by the use of isooctane in place of heptane (isopropanol-isooctane-1 N H_2SO_4 40:10:1) (v/v). After 2 hours the phases were broken by adding 18 ml of isooctane and 12 ml of H_2O. The phases were separated and the isooctane phase was washed with 15 ml of Borgström's alkaline ethanol (ethanol-H_2O-1 N NaOH 50:44:5) (v/v) (13). The alkaline aqueous phase from this wash was backwashed with 10 ml of isooctane and the latter was combined with the isooctane phase from the initial extraction. The pooled isooctane extracts were washed with alkaline ethanol, and the alkaline-ethanol washes were combined.

An aliquot of the pooled isooctane extracts was taken to dryness in a glass counting vial, diphenyloxazole in toluene (5 mg/ml) was added, and radioactivity was assayed using a Packard Tri-carb liquid scintillation spectrometer. The lipids of this fraction are designated neutral lipid.

The pooled alkaline-ethanol washes were acidified and the FFA were re-extracted into 10 ml of isooctane. One aliquot of this was titrated (5), and a second aliquot was taken to dryness for radioassay as described above. This fraction is designated tissue free fatty acids.

The extent to which phospholipid contributed to the radioactivity in the neutral lipid fraction was directly determined using column chromatography on silicic acid (14). Two grams of 325-mesh silicic acid (Bio-Rad Laboratories, Richmond, Calif.) was slurried into a glass column with chloroform. The material was activated by successive washes with 4 ml of diethyl ether, 12 ml of acetone-ether 1:1 (v/v), and 8 ml of diethyl ether. An aliquot of the neutral lipid fraction was added to the top of the column in 1 ml of chloroform, and elution was carried out with 80 ml of chloroform followed by 80 ml of methanol. In several experiments, at different levels of medium FFA, some with glucose in the medium and some without, the radioactivity in the chloroform eluate was found to account for 96.8% to 99.7% of the total radioactivity in the neutral lipid fraction.

FFA concentrations of the medium were measured by the method of Dole (5).

RESULTS

Effect of FFA Concentration. As shown in Table 1, the conversion of palmitate-1-C^{14} to neutral lipid increased with increasing concentrations of palmitate in the medium. The relation was not a regular one, perhaps because of the small number of experiments at each concentration, but the trend is clear. There was an eightfold increase in calculated incorporation as the medium concentration was raised from 0.12 up to 1.64 μeq/ml. The increases in oxidation of palmitate to $C^{14}O_2$ over this range of medium palmitate concentrations were of a similar magnitude. The total amounts of C^{14} appearing in $C^{14}O_2$ were similar to the amounts converted to neutral lipid.

TABLE 1. METABOLISM OF PALMITATE-1-C^{14} AT VARIOUS CONCENTRATIONS OF PALMITATE IN THE MEDIUM

Total Medium FFA	Medium Palmitate	Conversion to Neutral Lipid*	Conversion to $C^{14}O_2$*
μeq/ml	*μeq/ml*	*μeq/g/2 hrs*	*μeq/g/2 hrs*
0.23	0.12 (6)†	0.13 ± 0.02	0.07 ± 0.01
0.46	0.31 (5)	0.21 ± 0.01	0.33 ± 0.04
0.90	0.69 (3)	0.83 ± 0.20	0.52 ± 0.04
1.05	0.82 (6)	0.57 ± 0.01	0.39 ± 0.02
1.18	0.93 (3)	0.49 ± 0.06	0.54 ± 0.06
1.38	1.09 (3)	0.83 ± 0.06	0.71 ± 0.04
1.66	1.33 (5)	0.71 ± 0.08	0.56 ± 0.07
1.82	1.46 (3)	1.26 ± 0.08	0.81 ± 0.05
2.02	1.64 (3)	1.07 ± 0.03	0.87 ± 0.06

* Based on specific activity of medium palmitate determined as described under Methods. $\frac{\text{cpm in neutral lipid (or in } CO_2)}{\text{S. A. of medium palmitate}}$

† Numbers in parentheses indicate the numbers of pairs of tissues studied. The difference between the values at a medium concentration of 0.12 μeq/ml and the values at all higher concentrations are highly significant ($p < 0.001$).

The relative specific activity of the tissue FFA fraction at the end of the incubation at different initial concentrations of medium FFA is shown in Table 2, expressed as a percentage of the zero time specific activity of the total medium FFA (without regard to composition of the fatty acids). The specific activity of the tissue FFA was in all cases well below that of the medium FFA, but approached it more closely at

TABLE 2. CHANGES IN RELATIVE SPECIFIC ACTIVITY OF TISSUE FFA WITH VARYING FFA CONCENTRATIONS IN THE MEDIUM

Concentration of Medium Palmitate	Relative Specific Activity of Tissue FFA*
μeq/ml	*per cent*
0.12	3.3
0.68	4.0
0.92	9.8
1.09	18.8
1.46	22.6
1.64	18.2

* $\frac{\text{Final specific activity of tissue FFA}}{\text{Initial specific activity of medium FFA}} \times 100$

Specific activities are expressed in terms of total radioactivity per total titratable FFA without reference to fatty acid composition of the fractions.

TABLE 3. EFFECT OF EPINEPHRINE (1 μG/ML MEDIUM) ON PALMITATE UTILIZATION IN MUSCLE

Concentration of Medium Palmitate	Concentration of Medium Glucose	Number of Pairs of Muscles Studied	Conversion to Neutral Lipid*		Conversion to CO_2*		Relative Specific Activity of Tissue FFA†	
			Control	Epinephrine	Control	Epinephrine	Control	Epinephrine
μeq/ml	*mg/100 ml*		*μeq/g/2 hrs*		*μeq/g/2 hrs*		*per cent*	*per cent*
0.22	None	3			0.57 ± 0.08	0.46 ± 0.07	9 ± 1	8 ± 0.4
					($p < 0.5$)			
0.43	None	5	0.42 ± 0.02	0.43 ± 0.03	0.19 ± 0.01	0.14 ± 0.01	12 ± 1	12 ± 1
					($p < 0.005$)			
1.47	None	5	0.82 ± 0.05	0.78 ± 0.06	0.58 ± 0.11	0.51 ± 0.10		
					($p < 0.5$)			
0.14	77	3	0.20 ± 0.02	0.20 ± 0.03	0.12 ± 0.01	0.13 ± 0.01	4.2	4.2
0.89	77	3	0.95 ± 0.02	0.82 ± 0.13	0.47 ± 0.01	0.62 ± 0.06	29.7	29.5
0.89	71	6	0.91 ± 0.02	0.84 ± 0.03	0.26 ± 0.01	0.28 ± 0.02	21.7	18.6

The probability of differences between means occurring by chance has been evaluated by the (t) test in each series of paired muscles.
* Calculated as in Table 1 and described under Methods.
† As in Table 2.

higher initial medium concentrations. The important bearing of these findings on interpretation of the results shown in Table 1 is discussed below. It should be noted that the FFA in the medium at concentrations of 0.68 μeq/ml and above was 70% to 80% palmitic acid, whereas the FFA in the tissue almost certainly contained a lesser percentage of palmitic acid. It is likely that the specific activity of palmitic acid in the tissue FFA pool approached that of the medium more closely than suggested by the data in Table 2.

Effects of Epinephrine. The effect of epinephrine (1 μg/ml) on neutral lipid synthesis and on $C^{14}O_2$ production from palmitate-1-C^{14} was tested at low and at high concentrations of FFA in the medium. As shown in Table 3, there was no demonstrable effect at any concentration of FFA tested, either in the presence or in the absence of glucose.

The experiments measuring oxidation to CO_2 again showed no epinephrine effect in most cases. At an FFA concentration of 0.43 μeq/ml in the medium without glucose present, there was a 26% inhibition of CO_2 production that appeared statistically significant, but at the concentrations tested above and below this there was no significant effect.

Effects of Glucose. As shown in Table 4, glucose added to the medium markedly enhanced palmitate-1-C^{14} incorporation into neutral lipids at four different FFA concentrations. This glucose stimulation was of

TABLE 4. EFFECT OF GLUCOSE ON PALMITATE UTILIZATION IN MUSCLE

Concentration of Medium Palmitate	Concentration of Medium Glucose	Number of Pairs of Muscles Studied	Conversion to Neutral Lipid*		Conversion to CO_2*		Relative Specific Activity of Tissue FFA†	
			Control	Glucose	Control	Glucose	Control	Glucose
μeq/ml	*mg/100 ml*		*μeq/g/2 hrs*		*μeq/g/2 hrs*		*per cent*	*per cent*
0.14	82	3	0.07 ± 0.009	0.15 ± 0.009	0.07 ± 0.008	0.08 ± 0.02	3	3.4
			($p < 0.05$)					
0.93	73	3	0.49 ± 0.06	1.04 ± 0.06	0.54 ± 0.05	0.52 ± 0.04	9.6	10.7
			($p < 0.005$)					
1.11	82	3	0.80 ± 0.16	1.46 ± 0.22	1.63 ± 0.36	1.35 ± 0.32	17.3	13.4
			($p < 0.05$)				18.2	11.2
1.64	73	3	1.07 ± 0.18	2.06 ± 0.72	0.87 ± 0.06	0.76 ± 0.05		16.9
	43			0.70 ± 0.02		0.32 ± 0.02		
0.95		6‡		($p < 0.001$)				11.4
	104			0.96 ± 0.40		0.37 ± 0.02		

The probability of differences between means occurring by chance has been evaluated by the (t) test in each series of paired muscles.
* Calculated as in Table 1 and described under Methods.
† As in Table 2.
‡ Six pairs of muscles, one of each pair incubated at 43 mg/100 ml, the other at 104 mg/100 ml of glucose.

a similar magnitude (80% to 112% increase) and statistically significant ($p < 0.05$) at all four concentrations of FFA tested. On the other hand, the conversion of palmitate to CO_2 was either unchanged or, at higher concentrations of FFA, slightly decreased. This dissociation of effects indicates that the increase in neutral lipid synthesis is not attributable simply to an increased rate of penetration of labeled palmitate into the tissue but represents a true stimulation of glyceride deposition. This interpretation is further supported by the measurements of the tissue FFA specific activities which, as shown in Table 4, were not higher in the presence of glucose at any medium FFA concentration, and were actually lower at FFA concentrations of 1.11 and 1.64 μeq/ml.

Possible Effects of Interfibrillar Adipose Tissue. Since muscle is interlarded with adipose tissue, it is necessary to consider whether any of the effects observed in the intact muscle strips might be the result of changes in the metabolic patterns of such adipose tissue. The total triglyceride content of rat muscle was determined and found to be 17.5 mg/g wet weight of tissue; the triglyceride content of adipose tissue (epididymal fat pad) was 915 mg/g wet weight of tissue. If it is assumed that all of the triglyceride in the muscle represents interfibrillar adipose tissue, the amount of FFA that would be converted to CO_2 by that amount of adipose tissue can be estimated using the data of Milstein and Driscoll (15) on FFA oxidation by isolated adipose tissue. On this basis, an oxidation of 0.00015 μeq of medium palmitate per gram per hour is calculated; the observed rate of oxidation was 0.16 μeq/g/hour. It is clear that the adipose tissue present could not have contributed significantly to the observed palmitate oxidation. A similar calculation was made of the rate of triglyceride synthesis expected on the basis of the adipose tissue content, using data obtained with the epididymal fat pad incubated in glucose (16). The calculated rate was found to be one-third the observed rate. It is possible that the results obtained for effects on triglyceride synthesis are quantitatively distorted due to adipose tissue in the muscle, but they are not likely to be qualitatively altered. Furthermore, the fact that epinephrine, which has been shown to inhibit FFA incorporation into adipose tissue triglycerides (16), was without effect on incorporation into muscle triglycerides is against any major contribution of interlarded adipose tissue to the observed results.

DISCUSSION

The results of the present study suggest that a major factor governing the rate of lipid utilization in skeletal muscle is the concentration of available FFA in the serum. The rate of fatty acid oxidation to CO_2 and the rate of deposition of fatty acids in the form of triglycerides were both markedly increased as the FFA concentration in the medium was increased. Fritz and co-workers (11), using FFA concentrations up to approximately 0.6 μeq/ml, have previously reported similar findings with respect to palmitate oxidation by rat skeletal muscle. These workers, however, utilized very low concentrations of serum albumin and encountered variations in rates of oxidation with changes in fatty acid to albumin ratios. Reichl and Felt (17) have recently shown that at albumin concentrations of 1% or below, the uptake of fatty acids from the medium into adipose tissue is more rapid than it is at higher concentrations, and that glucose stimulation is less marked or not demonstrable at all. The present studies, utilizing physiological concentrations of serum albumin and covering a broader range of FFA concentrations, similar to those encountered in physiological and pathological states, supplement and extend the work of Fritz and co-workers.

The rate of conversion of labeled palmitate to $C^{14}O_2$ at the highest concentration studied here (1.64 μeq/ml) was more than ten times that at the lowest concentration (0.12 μeq/ml). These concentrations of palmitate corresponded to total FFA concentrations of 2.02 and 0.23 μeq/ml, respectively. If FFA utilization by the tissue *in vivo* is at all similarly sensitive to serum FFA concentration, it is clear that fluctuations in the concentration of this lipid component must strongly influence rates of lipid oxidation in muscle. Since muscle metabolism represents such a large fraction of the body's total metabolism, it would follow that overall metabolic rate may well be determined in large part by serum FFA levels, which in turn depend upon rates of mobilization from adipose tissue depots. For example, it is well established that serum FFA levels are, for reasons not clearly understood, consistently and strikingly elevated in hyperthyroidism (18). While this may only reflect accelerated mobilization, and thus be secondary to the prime cause of the hypermetabolism, an equally plausible hypothesis that we wish to suggest here is that the hypermetabolism is actually the direct result of the sustained high serum FFA levels, which in turn are caused by some more immediate metabolic effect of the thyroid hormone.

In a similar manner, the hypermetabolism observed after epinephrine injection may again be the direct result of the excessive mobilization of depot fat with consequent elevation of serum FFA levels. The conflicting findings regarding the calorigenic action of

epinephrine have been reviewed by Ellis (19), who concludes that the weight of evidence is against the notion that it is attributable to the increased cardiac work or to the hyperglycemia caused by epinephrine. Lundholm (20) has correlated the epinephrine-induced increases in oxygen consumption with hyperlacticacidemia. These earlier studies were done before the striking effect of catecholamines on FFA mobilization was recognized. The possibility, suggested by the present studies, that this FFA mobilization may be the immediate cause of the calorigenic action of epinephrine, deserves further investigation.

What we are proposing, in essence, is that the rate of utilization of lipid (FFA) is, to a large extent, simply controlled by the concentration of substrate available (blood FFA level) just as the rate of utilization of carbohydrate is controlled by blood glucose levels (21). In the case of glucose utilization, it is clear that hormonal control of uptake and utilization in the periphery strongly modulates the effects of substrate concentrations per se. Similar hormonal control of peripheral utilization of FFA may be demonstrated by further work, but at the moment the weight of evidence is that hormonal control is exercised primarily over the rate of mobilization, i.e., at the adipose tissue depots. Further efforts to establish *in vivo* the quantitative importance of serum FFA concentrations per se, as proposed here, appear to be justified.

The rates of incorporation of labeled palmitate into triglycerides rose with increasing medium FFA concentration in a manner similar to that observed for palmitate oxidation. This implies the possibility of net triglyceride deposition if the blood FFA are maintained at high levels over prolonged periods. The deposition of triglycerides in cardiac muscle observed by Maling and Highman (8), and in skeletal muscle by Feigelson *et al.* (7) after norepinephrine infusions, could be explained on this basis. In the present studies it has been shown that epinephrine added *in vitro* does not itself affect the rate of triglyceride synthesis from palmitate. Although other indirect effects of the catecholamines may contribute, it seems reasonable to attribute the lipid deposition observed in these *in vivo* studies mentioned above directly to the elevated FFA levels. In other words, the FFA mobilizing action of epinephrine acting on depot fat may be a sufficient explanation for the triglyceride deposition in muscle observed following its administration in large amounts. The possibility that the more marked accumulation of triglyceride in the liver during norepinephrine infusion (7) is also due to a direct effect of the elevated serum FFA levels is under study.

Oxidation of medium palmitate to CO_2 was not importantly influenced by the presence of physiological concentrations of glucose in the medium. These findings are in agreement with the earlier results of Fritz *et al.* (11). They supplement the conclusions of Allen *et al.* (22) and of Neptune *et al.* (3), based on studies of oxidation of endogenous tissue lipids, that glucose *in vitro* does not significantly alter the rate of oxidation of lipid. Recently Fritz and Kaplan (23) have shown that in diaphragm muscle taken from rats previously starved for 4 days, or from rats with chronic alloxan diabetes, the rate of palmitate oxidation is much higher than normal, and that addition of glucose to the medium returns it toward normal. Schwartzman and Brown (24), using diaphragms taken from rats starved for 24 hours, found no effect of glucose at a concentration of 150 mg/100 ml, but at 500 mg/100 ml there was a statistically significant inhibition of palmitate oxidation (approximately 25%). Under certain abnormal conditions, then, glucose can apparently influence the rate of fatty acid oxidation in muscle. More significant, however, is the agreement among the various groups that have studied the problem that in muscle tissue taken from normal, fed animals, and at physiological concentrations, glucose does not appear to alter the rate of oxidation of added long-chain fatty acids.

The physiologic effects of glucose on the relative rates of carbohydrate and lipid metabolism may then be limited to its effect on the rate of mobilization of depot fats, as suggested by Dole (5) and Gordon (6). Again, the serum FFA level appears to be a key controlling factor.

On the other hand, the rate of conversion of palmitate to neutral lipid was markedly enhanced. The interesting implication of this finding is that the endogenous stores of muscle lipid may be built up during periods when extra glucose is available (e.g., postprandially), to be drawn upon during periods when carbohydrate is less available. The studies of Volk *et al.* (2) first demonstrated the major role of endogenous lipid in oxidative metabolism of peripheral tissues. Later studies by Neptune *et al.* (3) indicate that diaphragm from fed rats contains sufficient lipid to support normal oxygen consumption *in vitro* for 4 hours, whereas the diaphragm from fasted rats has a very limited reserve of tissue lipid on which to draw. It should be noted, however, that the latter studies also indicate that much of the endogenous lipid available for oxidation is exhausted in 4 hours, even in the unstimulated diaphragm. Over any extended period of time, and certainly during exercise, the exogenous lipid brought to the tissue, primarily in the form of FFA, must be the major source of lipid substrate. Moreover, it remains to be

determined to what extent endogenous lipid would be utilized if FFA were simultaneously available.

Finally, it is important to call attention to a difficult question of interpretation arising in the present studies, as well as in the work of others using similar methods (11). The fraction of added C^{14}-palmitate appearing in the $C^{14}O_2$ or in labeled triglycerides depends not only on the rates of the reactions involved, but also on the specific activity of the immediate tissue precursors and intermediates. In the present studies the specific activity of the tissue FFA pool was measured at the end of the incubation. As shown in Table 2, the terminal specific activity was higher relative to that in the medium as the total initial medium FFA concentration was increased. This result would be anticipated as the result of a greater net transfer of labeled fatty acid into the tissue and through the tissue FFA pool en route either to CO_2 or neutral lipid. On the other hand, an effect of FFA concentration on the rate of equilibration between medium and tissue FFA cannot be ruled out. Further studies on this important point are needed before the observed isotope results can be considered conclusive.

REFERENCES

1. Andres, R., C. Cader and K. L. Zierler. *J. Clin. Invest.* **35**: 671, 1956.
2. Volk, M. E., R. H. Millington and S. Weinhouse. *J. Biol. Chem.* **195**: 493, 1952.
3. Neptune, E. M., Jr., H. C. Sudduth and D. R. Foreman. *J. Biol. Chem.* **234**: 1659, 1959.
4. Gordon, R. S., Jr., and A. Cherkes. *J. Clin. Invest.* **35**: 206, 1956.
5. Dole, V. P. *J. Clin. Invest.* **35**: 150, 1956.
6. Gordon, R. S., Jr. *J. Clin. Invest.* **36**: 810, 1957.
7. Feigelson, E. B., W. Pfaff and D. Steinberg. In preparation.
8. Maling, H. M., and B. Highman. *Am. J. Physiol.* **194**: 590, 1958.
9. Gordon, R. S., Jr., and A. Cherkes. *Proc. Soc. Exptl. Biol. Med.* **97**: 150, 1958.
10. Umbreit, W. W., R. H. Burris and J. F. Stauffer. *Manometric Techniques and Tissue Metabolism.* Minneapolis, Burgess Publishing Co., 1957.
11. Fritz, I. B., D. G. Davis, R. H. Holtrop, and H. Dundee. *Am. J. Physiol.* **194**: 379, 1958.
12. Steinberg, D. *Anal. Biochem.* **1**: 23, 1960.
13. Borgström, B. *Acta Physiol. Scand.* **25**: 111, 1952.
14. Borgström, B. *Acta Physiol. Scand.* **25**: 101, 1952.
15. Milstein, S. W., and L. H. Driscoll. *J. Biol. Chem.* **234**: 19, 1959.
16. Steinberg, D., M. Vaughan and S. Margolis. *J. Biol. Chem.* **235**: PC 38, 1960.
17. Reichl, D., and V. Felt. *Metabolism* **9**: 807, 1960.
18. Rich, C., E. L. Bierman and I. L. Schwartz. *J. Clin. Invest.* **38**: 275, 1959.
19. Ellis, S. *Pharmacol. Revs.* **8**: 485, 1956.
20. Lundholm, L. *Acta Physiol. Scand.* **19**: **Suppl. 67**: 1, 1949.
21. Soskin, S., and R. Levine. *Am. J. Physiol.* **120**: 761, 1937.
22. Allen, A., B. Friedmann and S. Weinhouse. *J. Biol. Chem.* **212**: 921, 1955.
23. Fritz, I. B., and E. Kaplan. *Am. J. Physiol.* **198**: 39, 1960.
24. Schwartzman, L. I., and J. Brown. *Am. J. Physiol.* **199**: 235, 1960.

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Metabolism of stearate-1-C^{14} in the isolated cow's udder*

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SUMMARY

One-half of a lactating cow's udder was perfused with heparinized and oxygenated cow's blood at 38° for 150 minutes; 30 minutes after the onset of the perfusion, approximately 200 μc of stearate-1-C^{14} (albumin-bound) was added to the perfusing blood, together with inactive acetate and glucose. Almost all the blood's stearate-1-C^{14} was absorbed by the gland, and much of the added C^{14} was recovered from the glycerides of the udder tissue. Stearic acid and oleic acid of the glycerides showed significant specific activities. Negligible activities were detected in the shorter chain fatty acids of the glycerides, in citric acid, and in CO_2, indicating that stearic acid was broken down to only a very small extent.

It has been known for several years that part of the milk fat of the ruminant is derived as a result of lipogenesis in the mammary gland itself (short-chain fatty acids) and that the other part originates from plasma lipid fatty acids (C_{18} fatty acids) (1).

Glascock *et al.* (2) administered tracer doses of tritium-labeled stearate, either free or combined, by mouth to lactating cows and goats and noted an efficient transfer to the milk. The total recovery amounted, in one experiment, to 60%. Radioactivity was detected in milk fat within 4 hours after the administration, and the specific activity reached a maximum after 23 hours. In the glyceride fatty acids the highest specific activity was observed in the nonvolatile fractions. The specific activity of the stearic acid was estimated to be approximately three times that of oleic acid, indicating a very considerable conversion of one acid to the other. The lower fatty acids of the glycerides showed only a very low activity, indicating that stearic and oleic acids are broken down to a very slight extent. This agrees with the view that these lower acids are not derived from the degradation of the long-chain acids. Glascock *et al.* (3) did not succeed in identifying the plasma lipid fraction which was most important for the transport of the labeled stearic acid from the digestive tract to the udder.

As Fredrickson and Gordon (4) state in a recent review, free or unesterified long-chain fatty acids (FFA) are present in the plasma of man and the common laboratory mammals in concentrations in the range of 0.2 to 2.0 mEq/liter under normal physiological conditions. These acids are primarily bound to albumin, and the rate of transfer of FFA *in vivo* is considerable, as the average fatty acid molecule remains in the plasma for only a few minutes at a time. Garton *et al.*[1] have found that the FFA of cow plasma consists mostly of stearic and oleic aids, together with palmitic and palmitoleic acids.

In the experiments reported here, we studied the metabolism of albumin-bound stearic acid-1-C^{14} by the perfused cow's udder. A preliminary report of this work has been published (5).

METHODS

Perfusion of Half Udder. The perfusion experiment was carried out on one lactating half udder from a cow yielding 15 liters of milk daily. The half udder was perfused for 150 minutes by the method of Peeters and Massart (6). The experimental details of the perfusion technique and $C^{14}O_2$ collection have been described previously (7). Immediately before slaughter the cow was milked out as completely as possible after an intravenous injection of oxytocin. The udder was removed as soon as the cow was shot, and the gland was completely bisected along the median septum and one-half

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[1] G. A. Garton, A. K. Lough, and W. R. H. Duncan. Private communication.

connected to the perfusion apparatus. Heparinized, fresh cow's blood (8.75 liters) was used for the perfusion of this half udder. For the preparation of stearic acid bound to blood plasma proteins we followed essentially the method described by McCalla *et al.* (8). Stearic acid-1-C^{14} (0.5 mc = 6.56 mg) was dissolved in 1 N NaOH on a steam bath. The solution was then added to 50 ml of saline. The day before the experiment, fresh heparinized cow's blood was centrifuged and the plasma collected. The dissolved stearate was added to 200 ml of plasma, shaken, and left overnight in the refrigerator. The following day the plasma was centrifuged to remove the precipitate. The plasma thus prepared contained approximately 200 μc of stearic acid-1-C^{14} bound to plasma proteins. This solution was added to the blood 30 minutes after the beginning of the perfusion. After the addition of the labeled substrate, a solution containing 10 g of $CH_3COONa \cdot 3\ H_2O$ and 2 g of glucose dissolved in 300 ml of water was added to the perfusion blood by means of a constant-drip device at the rate of 2.5 ml per minute. Then, 115 minutes after the addition of the isotope, 10 I.U. of oxytocin was injected intra-arterially and 78 ml of milk was collected. After perfusion, the udder was removed from the apparatus and the skin, teats, and adipose tissue were removed by dissection. The udder tissue was weighed (4.74 kg), cut into pieces, and frozen at $-15°$ for 1 hour. Blood samples were taken before and after the perfusion experiment.

Analysis of the Blood Samples. The packed cell volume (hematocrit) was measured. The total lipids were extracted from the plasma with chloroform-methanol. The total fatty acids were determined following hydrolysis of the total lipids with 6 N HCl in methanol (9). Free and total cholesterol were estimated in the plasma before and after perfusion according to the method of Schoenheimer and Sperry (10).

Isolation of Casein, Lactose, and Citric Acid from Milk. Casein was precipitated from the skim milk by acidification to pH 4.5 (7). The whey was lyophilized and lactose isolated from the dried powder as described by Reiss and Barry (11). The solutions from the lactose crystallization were evaporated to dryness, dissolved in 20 ml of water, and passed through a Dowex 1 ($HCOO^-$ form) column (2 × 20 cm). The citric acid eluted from this column was rechromatographed on silicic acid as described previously (7).

Isolation of Udder-Tissue Fat and Milk Fat Fractions. Total lipid was isolated from udder tissue and milk as described previously (7). Total milk fat was not fractionated further. Lower FFA were removed from udder-tissue fat by washing the ether extract several times, first with 1% Na_2CO_3, then with water. The technique of chromatography on silicic acid, originally developed by Fillerup and Mead (12) and described in detail by Garton and Duncan (13) for the separation of the plasma lipids of lactating cows, was applied to the separation of udder-tissue fat. Columns of 5 cm diameter, containing a mixture of silicic acid and Hyflo Super-Cel 200/70 (g/g) and having a capacity of separating about 6 g of total fat, were used. In the eluate, free cholesterol and esterified cholesterol were estimated according to Kenny (14), phospholipids by the phosphorus determination method of Allen (15), and glycerides by the hydroxamic acid method of Morgan and Kingsbury (16). Separation of the cholesterol esters, glycerides, free cholesterol, and phospholipids was obtained as shown in Figure 1. Evidence was obtained that on these columns the higher FFA were spread mainly over the glyceride and free cholesterol fractions. The material in the cholesterol ester peak, the glyceride peak, and the free cholesterol peak

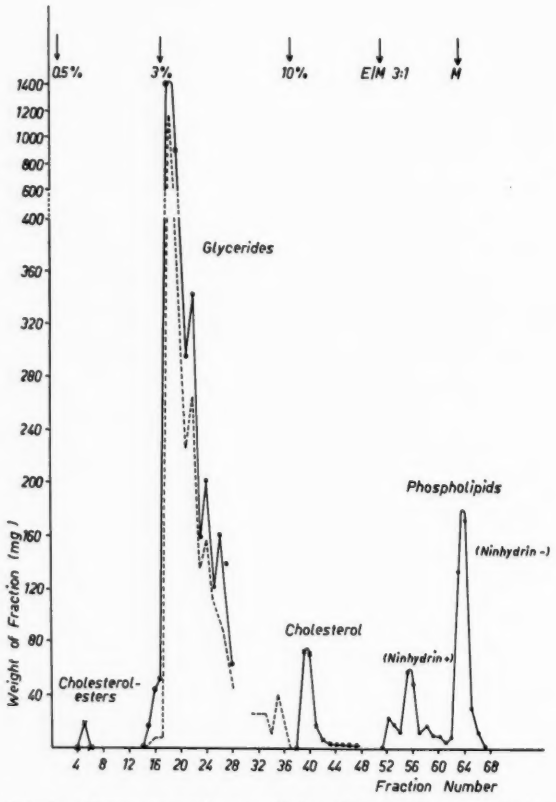

FIG. 1. Chromatography on silicic acid of udder-tissue lipids. Solvents used were 0.5%, 3%, and 10% ethyl ether-petroleum ether, ether-methanol 3:1 (v/v), and methanol. The solid lines of the glyceride peak indicate weight determinations; the dotted lines indicate hydroxamic acid determinations.

was taken up into wet ether. A large amount of nonlabeled stearic acid was added to each peak and the fraction passed through two successive Dowex 1 (OH^- form) columns. These columns had been previously saturated with wet ether (17). Ten milliliters of Dowex 1 (OH^- form) was used per mM of added fatty acid. The neutral fat fractions were eluted with wet ether. Elution with wet ether was stopped when no further material came off the columns. The FFA were then eluted with a mixture of ether–95% ethanol-sulfuric acid 200:8:17 (v/v). Preliminary experiments on Dowex 1 (OH^- form) columns with a mixture of inactive tripalmitin and highly labeled stearic acid showed that under these conditions no contamination of the neutral fat fraction with FFA occurred. However, some hydrolysis, occurring during the passage of the neutral fat fraction through the Dowex columns, could not be avoided. Finally the free cholesterol fraction was treated with digitonin; the digitonide was decomposed in benzene, and the free cholesterol isolated.

Chromatographic Separation of Fatty Acids. The main fraction of glycerides was saponified, acidified, and the even-numbered fatty acids were isolated by the reversed phase chromatography technique of Howard and Martin (18), as described previously (7). Odd-numbered fatty acids are not detected by this technique as they occur only in trace amounts in udder and milk lipids.

Radioactivity Measurements. The radioactivity measurements were made with a windowless flow counter. After wet combustion, lactose and casein were counted as $BaCO_3$; citric acid and the sodium salts of the individual fatty acids were counted as previously described (7). The magnitude of the counting rates obtained and the thickness of the material were of the same order as those described by Verbeke *et al.* (7).

Cholesterol esters, cholesterol, glycerides, phospholipids, whole blood, and blood plasma samples were taken at the end of the perfusion experiment. They were plated on plastic disks and counted at infinite thickness until 10,000 counts.

RESULTS

Perfusion. In the experiments we carried out with udders from slaughterhouse cows, the O_2 uptake of the isolated glands in the course of the first 2 hours of perfusion compares rather favorably with the activity of the gland *in vivo*. However, for reasons which at present are but partially clear, the physiological activity of the excised glands declines rather quickly afterward. We therefore limit our perfusion experiments to 2 hours. The milk production in the experiment reported here was somewhat lower than that of most of our earlier studies.

Blood Samples. In view of the changes in plasma volume which result from the addition of water to the blood by way of drip infusion, the concentrations of lipid constituents in the plasma following perfusion were corrected according to the hematocrit to make them comparable with values on the plasma before perfusion. Determination of the total fatty acid content of the plasma before and after perfusion gave values of 1,946 mg/liter and 1,862 mg/liter, respectively. The figures for total cholesterol were 1,376 mg/liter before, and 1,456 mg/liter after perfusion, and for free cholesterol, 274 mg/liter before, and 290 mg/liter after perfusion. The concentrations of these lipid constituents in the plasma at the end of perfusion are not considered significantly different from the preperfusion control. At the end of the perfusion no C^{14} was detected in the red cells, and only 2 μc was present in the total plasma, indicating that virtually all the added stearate had been removed.

Carbon Dioxide. In total, 0.35 μc of $C^{14}O_2$ was collected. This amount is very small compared with previous perfusion experiments performed in the presence of C^{14}-labeled lower fatty acids, and indicates the small extent of catabolism of the added substrate.

Milk Constituents. No detectable incorporation of C^{14} was observed in casein or lactose. Milk citric acid showed a small but definite C^{14} incorporation (0.05 $m\mu c$/mg carbon). In total, 0.002 μc was recovered in this fraction.

Milk fat showed a specific activity of 0.007 $m\mu c$/mg. In total, 0.06 μc was recovered in this fraction.

Udder-Tissue Fat. Table 1 gives the specific activities of the different fat fractions, before and after pas-

TABLE 1. SPECIFIC ACTIVITIES OF LIPID FRACTIONS OF UDDER TISSUE BEFORE AND AFTER PASSAGE THROUGH TWO SUCCESSIVE DOWEX 1 (OH^-) COLUMNS

Fat Fraction	Weight	Specific Activity*		
		Before Passage	After Passage Through	
			1st Dowex Column	2d Dowex Column
	per cent			
Cholesterol esters	0.45	†	†	357 ± 36
Glycerides	83.5	1220 ± 122	834 ± 83	827 ± 80
Free cholesterol	3.25	612 ± 61	202 ± 20	210 ± 21
Phospholipids 1‡	4.5	347 ± 35		
Phospholipids 2‡	8.0	653 ± 65		

* Cpm infinitely thick. Means of triplicates ± standard error.
† Not determined.
‡ The phospholipid fractions were not passed through Dowex columns.

sage through two successive Dowex 1 (OH^- form) columns. A sharp drop in activity was observed after passage of the glyceride fraction and the free cholesterol fraction through a first Dowex column, due to removal of the FFA. Passage of either of the neutral fat fractions through a second Dowex column did not alter the specific activity, indicating a complete removal of the FFA after passage through the first Dowex column. Although a sharp drop in activity of the cholesterol peak was observed after passage through the Dowex columns, some activity still remained, which we ascribed to the presence of traces of diglycerides. No attempt was made to distinguish the partial glycerides. After precipitation of the cholesterol as digitonide, followed by decomposition, no activity could be detected in this fraction. Because of the presence of unknown amounts of deterioration products, derived from the resin after elution with the acid-ethanol mixture, no accurate value for the specific activity of the FFA fraction was obtained.

Table 2 gives the specific activities of the even-numbered glyceride fatty acids from udder tissue. The highest specific activity was observed in stearic acid followed by oleic acid, the specific activity of stearic acid being approximately 2.3 times that of oleic acid. All the other acids showed very low activities. The activities of the individual fatty acids are expressed as a percentage of total fatty acid activity. More than 50% of the total fatty acid activity is observed in the oleic acid fraction. From the specific activity of the crude fat, and from the total lipid present in the udder tissue, it was calculated that 166 μc was recovered in the total fat of the udder tissue. In an analogous manner we found that approximately 87 μc was incorporated into the glycerides, 7 μc into the phospholipids, and 0.2 μc into the cholesterol. These figures indicate that approximately 72 μc was probably present in the FFA.

TABLE 2. INCORPORATION OF C^{14} IN EVEN-NUMBERED FATTY ACIDS OF UDDER TISSUE GLYCERIDES

Fatty Acid	Specific Activity	Distribution in Glycerides as Molar Per Cent	Percentage of C^{14}*
	mμc/mM		
Butyric, Caproic, Caprylic	2.38 ± 0.36	17.72 ± 0.18	0.47 ± 0.09
Capric	4.32 ± 0.60	1.62 ± 0.03	0.08 ± 0.01
Lauric	3.74 ± 0.58	2.17 ± 0.04	0.09 ± 0.02
Myristic	3.19 ± 0.50	10.2 ± 0.1	0.37 ± 0.07
Palmitic	3.84 ± 0.19	25.21 ± 0.25	1.08 ± 0.09
Stearic	365 ± 13	11.1 ± 0.1	45.4 ± 3.4
Palmitoleic	3.26 ± 0.58	2.62 ± 0.05	0.09 ± 0.02
Oleic	159.2 ± 1.1	29.4 ± 0.3	52.4 ± 2.4

All values expressed as means ± standard errors.

* C^{14} recovered expressed as percentage of total fatty acid activities.

DISCUSSION

It is clear that albumin-bound stearate is removed from the circulating blood by the udder. Two hours after the start of perfusion, virtually all the labeled material had disappeared from the blood. Unfortunately, no greater number of blood samples was collected in the course of the experiment, though it is highly probable that the low concentration of C^{14} was reached at a much earlier moment. After intravenous injection of palmitic acid-1-C^{14}, bound to serum albumin into rats, Bragdon and Gordon (19) observed a very rapid disappearance of 99% of the labeled FFA from the circulating blood. The long persistence of the remaining 1% indicated that an exchange occurred between plasma FFA and the fatty acids of a tissue pool. As our results seem to indicate that an important activity was present in the FFA fraction isolated from udder tissue, a similar exchange as observed by Bragdon and Gordon (19) in the rat might have occurred.

A large percentage of the labeled substrate, absorbed from the blood, was metabolized and incorporated into the glycerides and phospholipids of the udder fat. On the basis of the respective specific activities and concentrations of these fractions in the udder fat, it is clear that most of the esterified stearate-1-C^{14} was present in the glycerides. Although no detailed analytical work was carried out on the fat of the secreted milk, it is likely that the milk fat activity distribution resembled that of udder fat. It would be desirable to investigate this question in another perfusion experiment carried out for a longer period under physiological conditions so as to obtain a higher specific activity in the secreted milk fat than we obtained in this experiment. In previous experiments carried out in the presence of C^{14}-labeled substances, it was always observed that C^{14} was distributed in an analogous manner between the fatty acids isolated from milk and from udder tissue. The specific activities in the milk fat fractions, however, are always much lower than those of the udder fat because of the limited duration of the perfusion experiments. It is to be noted, however, that the fatty acid pattern (i.e., the ratio stearic to oleic acid) of the milk fat can be significantly different from that of the udder fat.

Stearic acid was dehydrogenated to a considerable extent, giving rise to oleic acid. This dehydrogenation

must be a very active process as it occurred to over half of the stearic acid incorporated into the udder glycerides during the relatively short time of perfusion. Using stearic acid with the label on the 1 position, it is clear that the label would not be altered in the dehydrogenation. The unequivocal position of the label, the short experimental period, and the use of an isolated organ system characterize the dehydrogenation process much more clearly than that observed in the studies of Glascock *et al.* (2), where the tritium label was not uniformly positioned and the labeled stearate was administered by mouth. It was pointed out by these authors that a considerable degree of randomization and transformation of lipids takes place during digestion and absorption if the labeled material is administered by mouth, and this may lead to difficulties in the interpretation of milk fat radioactivities. Very little breakdown of the C_{18} acids to small molecules (acetyl-CoA) took place in the perfusion experiment, as is indicated by the very low specific activities detected in citric acid, the lower fatty acids, and CO_2, and by the absence of activity in the casein fraction. The specific activities of these last fractions were very much below those observed previously in the presence of C^{14}-acetate, -butyrate, -hexanoate and -isovalerate (20, 21). It is to be noted that Glascock *et al.* (2), in agreement with our observations, found a much higher specific activity in the long-chain fatty acids of milk glycerides than in the short-chain acids. The specific activities detected in palmitic acid and palmitoleic acid of the perfusion experiment deserve some further consideration. The direct conversion of stearic to palmitic acid would probably not be detected with the carboxy-labeled stearic acid used in these studies. It therefore appears probable that C^{14} was introduced into palmitic acid by way of acetyl-CoA, and it seems reasonable to assume that palmitic acid was dehydrogenated, giving rise to palmitoleic acid. Therefore it would be of interest to study the metabolism of C^{14}-labeled palmitic acid in another perfusion experiment.

Glascock (1) did not observe an especially high activity in the FFA fraction of the plasma of the cow in his experiments. He found that a highly active fraction, amounting to about 1% of the total plasma lipids, was precipitated with the phospholipids using acetone. According to Glascock (1), this fraction, which was not identified definitively, behaved chromatographically like neutral fat.

Several workers have observed that lipid was lost from the blood during its passage through the udder (22, 23, 24). Voris *et al.* (25) found that it was mainly the triglyceride fraction which was absorbed by the udder. Lough *et al.* (26) found that esterified higher fatty acids were taken up by the perfused cow's udder from the plasma, and always noted a loss of triglycerides. In some of their experiments the plasma content of both esterified and free cholesterol decreased, whereas in other experiments it increased during perfusion. In the stearate-1-C^{14} experiment, however, only a small decrease of the total fatty acid content of the plasma was observed during perfusion. The difference in total fatty acids before and after perfusion amounted to only 84 mg/liter of plasma, and this value was well within the limits of experimental error. In this experiment no determination of plasma triglycerides was carried out. Glascock (1) infused a tritium-labeled triglyceride emulsion in a lactating cow. The emulsion was "unphysiological," and caused acute symptoms because of agglutination of fat particles. However, 20% of the activity injected was removed in the milk fat, showing that circulating triglycerides can be used as precursors for the synthesis of milk fat. Riis *et al.* (27) infused cow plasma containing P^{32} and C^{14}-labeled lipids and proteins into a lactating cow. Little radioactivity was found in the expired CO_2, and none was found in the plasma volatile acids. They found only a trace of activity in casein and lactose, but significant activity was found in the butter fat.

The results reported in this paper show that circulating albumin-bound stearic acid can be used as a precursor for the synthesis of milk fat. As noted by Fredrickson and Gordon (4), FFA serve in men and the common laboratory animals during fasting as a transport form of fatty acids, being added to the blood mainly by peripheral adipose tissue and removed as blood perfuses the viscera. FFA may be considered as a major source of energy in the fasting state. The contribution of incoming chyle FFA to total plasma turnover would be very small. FFA-C^{14} appeared in the expired air as $C^{14}O_2$, and was incorporated into triglycerides and phospholipids of tissues.

It may well be that plasma FFA are of significance in the formation of milk fat glycerides, though physiological factors involved in the living cow remain to be determined. The hypothesis could be formulated that the FFA fraction would be of great importance quantitatively in the process of milk fat synthesis of the ruminant in the fasting state, but less important in normal circumstances.

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REFERENCES

1. Glascock, R. F. *Proc. Roy. Soc.* (*London*) *B.* **149:** 402, 1958.
2. Glascock, R. F., W. G. Duncombe and L. R. Reinius. *Biochem. J.* **62:** 535, 1956.
3. Glascock, R. F., D. J. McWeeny and R. W. Smith. Proceedings, Unesco Conference on Radioisotopes in Scientific Research. Paris. Paper 116, 1957.
4. Fredrickson, D. S., and R. S. Gordon, Jr. *Physiol. Revs.* **38**: 585, 1958.
5. Lauryssens, M., R. Verbeke, G. Peeters, G. A. Garton, A. K. Lough, and W. R. H. Duncan. *Arch. intern. physiol. et Biochem.* **68:** 511, 1960.
6. Peeters, G., and L. Massart. *Arch. intern. pharmacodynamie* **74:** 83, 1947.
7. Verbeke, R., M. Lauryssens, G. Peeters, and A. T. James. *Biochem. J.* **73:** 24, 1959.
8. McCalla, Ch., H. S. Gates, Jr. and R. S. Gordon, Jr. *Arch. Biochem. Biophys.* **71:** 346, 1957.
9. Böttcher, C. J. F., F. P. Woodford, E. Boelsma-Van Houte, and C. M. Van Gent. *Rec. trav. chim.* **78:** 794, 1959.
10. Schoenheimer, R., and W. M. Sperry. *J. Biol. Chem.* **106:** 745, 1934.
11. Reiss, O. K., and J. M. Barry. *Biochem. J.* **55:** 783, 1953.
12. Fillerup, D. L., and J. F. Mead. *Proc. Soc. Exptl. Biol. Med.* **83:** 574, 1953.
13. Garton, G. A., and W. R. H. Duncan. *Biochem. J.* **67:** 340, 1957.
14. Kenny, A. P. *Biochem. J.* **52:** 611, 1952.
15. Allen, R. J. L. *Biochem. J.* **34:** 858, 1940.
16. Morgan, D. M., and K. J. Kingsbury. *Analyst* **84:** 409, 1959.
17. Cason, J., G. Sumrell and R. S. Mitchell. *J. Org. Chem.* **15:** 850, 1950.
18. Howard, G. A., and A. J. P. Martin. *Biochem. J.* **46:** 532, 1950.
19. Bragdon, J. H., and R. S. Gordon, Jr. *J. Clin. Invest.* **37:** 574, 1958.
20. Lauryssens, M., R. Verbeke, G. Peeters, and A. Donck. *Biochem. J.* **73:** 71, 1959.
21. Lauryssens, M., R. Verbeke, G. Peeters, and M. Th. Reinards. *J. Dairy Research* **27:** 151, 1960.
22. Graham, W. R., Jr., T. S. G. Jones and H. D. Kay. *Proc. Roy. Soc.* (*London*). *B.* **120:** 330, 1936.
23. Maynard, L. A., C. M. McCay, G. H. Ellis, A. Z. Hodson, and G. K. Davis. *Cornell Univ., Agr. Expt. Sta. Mem.* **211:** 1, 1938.
24. Shaw, J. C., and W. E. Petersen. *J. Dairy Sci.* **23:** 1045, 1940.
25. Voris, L., G. Ellis and L. A. Maynard. *J. Biol. Chem.* **133:** 491, 1940.
26. Lough, A. K., W. R. H. Duncan, G. A. Garton, and G. Peeters. In *Biochemistry of Lipids*, edited by G. Popják, Oxford, Pergamon Press, 1960, p. 64.
27. Riis, P. M., J. R. Luick and M. Kleiber. *Am. J. Physiol.* **198:** 45, 1960.

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The role of the liver and of extrahepatic tissues in the transport and metabolism of fatty acids and triglycerides in the dog*†

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SUMMARY

Labeled free fatty acids (FFA) and chylomicron triglycerides were injected intravenously into intact and hepatectomized dogs. Measurements were then made of the rate of their removal from the circulation, conversion to other circulating lipids, oxidation, and distribution in certain tissues. Hepatectomy reduced the rate of removal of FFA from the circulation moderately, but almost abolished the appearance of radioactivity in circulating triglycerides, indicating that the liver is the chief site of conversion of plasma FFA to plasma triglycerides. Experiments in dogs with thoracic duct fistulae showed that these triglycerides enter the circulation through the hepatic sinusoids. In hepatectomized animals a small quantity of triglycerides continues to enter the blood stream, probably from the intestinal mucosa by way of the thoracic duct, and possibly from other sites as well. Hepatectomy or temporary exclusion of the liver from the circulation reduced the rate of removal of chylomicron triglycerides variably. Hydrolysis to form circulating FFA occurred as in intact dogs. Most of the triglycerides removed from the circulation of hepatectomized dogs appeared to enter adipose tissue. Evidence was obtained that these triglycerides were hydrolyzed prior to entrance of their constituent fatty acids into adipose tissue cells.

Chylomicron triglycerides and FFA[1] bound to albumin have been shown to have rapid turnover rates in the blood (1). The liver is known to play an important role in their removal from the circulation. Bragdon and Gordon (2) injected fasting rats intravenously with albumin-bound palmitic acid-1-C^{14} and with chylomicrons obtained from the thoracic duct lymph of rats fed palmitic acid-1-C^{14}. Ten minutes after the injection they found that 12% and 21%, respectively, of the radioactivity cleared from the blood was present in liver lipids. Removal of these lipid moieties from the circulation by the liver has also been demonstrated by measurement of portal-hepatic venous differences (3, 4, 5).

In the present investigation we studied the metabolism of chylomicron triglycerides and FFA in intact and hepatectomized dogs. The results obtained provide further information regarding the role of the liver, and emphasize the importance of extrahepatic tissues in the transport and metabolism of these constituents.

METHODS

Preparation of Animals. Male mongrel dogs, weighing 20 to 24 kg, which had fasted overnight, were anesthetized with sodium pentobarbital given intravenously in a dose of 30 mg per kilogram body weight. A side-to-side portocaval anastomosis was created, and a large polyethylene cannula was inserted into the inferior vena cava as described by Dakin *et al.* (6). Respiration was maintained with a pump connected to an endotracheal tube. The liver was then either excised or excluded from the circulation temporarily by placing ligatures around the portal vein and hepatic

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[1] Abbreviations: FFA = free fatty acids; TGFA = triglyceride fatty acids.

artery, and about the caval catheter above and below the liver. After hepatectomy and during exclusion of the liver, the animals were given a constant infusion of glucose, 0.1 g per kilogram body weight per hour, through a catheter placed in a femoral vein. Blood samples were taken and blood pressure measured through a catheter placed in the abdominal aorta via a femoral artery. Expired air was collected in polyvinyl beach balloons from the outlet of the respiration pump.

Preparation of Lipids for Injection. Palmitic acid-1-C^{14}, specific activity 5 mc per millimole, was complexed with lipoprotein-free proteins from dog serum as described previously (1). Chylomicrons in which the triglycerides were labeled in the fatty acid moiety were isolated from thoracic duct lymph of dogs fed palmitic acid-1-C^{14} in cream as described previously (1), except that the thoracic duct was cannulated directly with a plastic catheter. The radioactive preparations were injected intravenously over a period of 10 to 15 seconds in a volume of 10 to 20 ml.

Analytical Methods. Blood samples were mixed with 1 mg of sodium oxalate per milliliter, chilled immediately in ice water, and centrifuged at 3°. Lipids were extracted from plasma by the method of Davis (7) to provide an extract practically free of phospholipids.[2] In experiments using chylomicrons and for analysis of thoracic duct lymph, the samples were centrifuged at 140,000 × *g* (maximum) for 15 hours at 10°; the supernatant very low density lipoproteins, including chylomicrons, were then recovered with the aid of a tube slicer. The lipids were extracted from these lipoproteins in chloroform-methanol 2:1 (v/v); after at least 30 minutes the extract was equilibrated with one-fifth volume of water. Lipids were extracted from the infranatant plasma by the method of Davis (7). Adipose tissue lipids were extracted with at least 25 volumes of ethanol-acetone 1:1 (v/v) in a Waring Blendor for 1 minute. Lipids from other tissues were extracted for 15 hours with the same solvent in a Sohxlet apparatus. FFA were separated from neutral lipids by the method of Borgström (9), and titrated by Dole's procedure (10). In the case of adipose tissue, the separation was performed twice prior to analysis of FFA. Cholesterol esters were separated from glycerides on silicic-acid columns containing one-third celite by weight. Cholesterol esters were eluted with heptane-diethyl ether 100:2 (v/v), and glycerides and cholesterol with chloroform. Glyceride glycerol was determined by Carlson's modification of the Lambert-Neish method (11). C^{14} assays were carried out in a Packard liquid scintillation spectrometer, with 0.3% diphenyloxazole in toluene as the phosphor. Carbon dioxide was collected from the samples of expired air in a methanolic solution of hyamine and assayed for CO_2 and $C^{14}O_2$ as described by Fredrickson and Ono (12). The volume of air was recorded with a Collins' spirometer. Plasma glucose concentration was measured by a glucose oxidase method.[3] For convenience in comparing the radioactivity of TGFA with that of FFA, the former were calculated as three times the molar concentration of glyceride glycerol.

[2] Extracts made by this method from serum of dogs, rabbits, and healthy humans uniformly contain less than 5 mg of phospholipids per 100 ml of serum. Glyceride-glycerol values are usually 5% to 10% lower than those obtained by the method of Van Handel and Zilversmit (8).

RESULTS

Free Fatty Acid Metabolism. When palmitic acid-1-C^{14} was injected intravenously into fasted intact dogs, radioactivity disappeared exponentially from the plasma FFA, with a half time of about 2 minutes for approximately 15 minutes, after which the rate slowed. In three studies the specific radioactivity of expired CO_2 was maximal in about 15 minutes, and 22% to 34% of the C^{14} injected was found in expired CO_2 in 3 hours. Radioactivity appeared rapidly in plasma neutral lipids. In preliminary experiments practically no radioactivity was found in cholesterol esters separated from the other neutral lipids on silicic-acid columns. Therefore radioactivity in neutral lipids was assumed to be contained exclusively in TGFA. The specific activity of TGFA exceeded that of FFA after 30 minutes (Fig. 1).

When these studies were performed on four hepatectomized dogs, the specific activity-time curves for FFA were similar to those observed in intact dogs, except that the initial half times of disappearance were 2.5 to 3.5 minutes. In these animals specific activity of expired CO_2 was maximal in about 15 minutes, and 10% to 16% of the C^{14} injected was found in expired CO_2 in 3 hours. Very little radioactivity appeared in plasma TGFA of the four dogs during the first hour; in two of the animals, however, considerably more radioactivity was present after 2 hours (Fig. 2). In one of these animals an increase in plasma triglyceride concentration was observed as well. The specific activity of TGFA exceeded that of FFA in both of these animals after about 3 hours. Systolic blood pressure ranged from 70 to 110 mm Hg. Plasma glucose concentrations varied from 40 to 200 mg/100 ml, but were relatively stable in individual animals. Plasma FFA concentra-

[3] Glucostat reagent, Worthington Biochemical Corporation, Freehold, N.J.

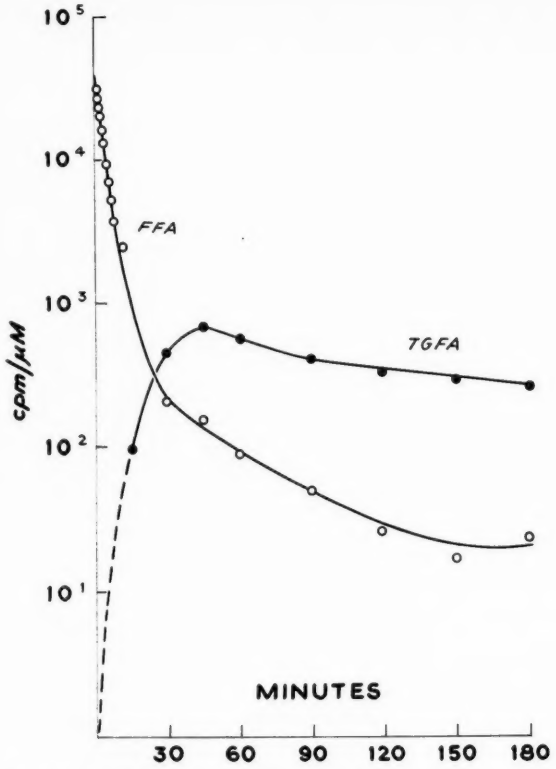


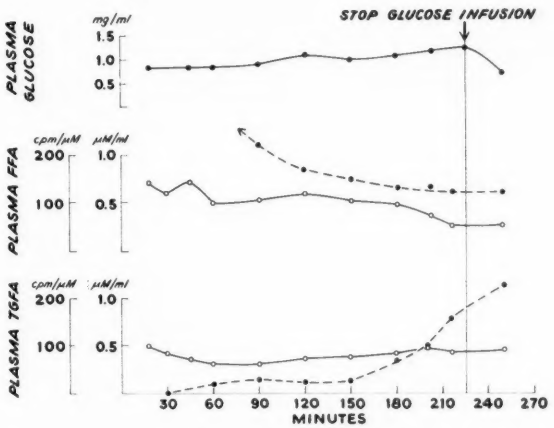
FIG. 1. Specific activities of FFA and TGFA of plasma after intravenous administration of palmitic acid-1-C^{14}, 3.8 × 10^7 cpm, to a fasted dog.

tions were in the range of 0.2 to 0.8 μmole per milliliter in three of the four dogs. In the fourth, plasma FFA concentrations were 0.94 to 1.46 μmoles per milliliter, and plasma glucose concentrations, 0.40 to 0.55 mg per milliliter.

The thoracic ducts of two intact dogs that were fed cream 1 hour prior to operation were cannulated, and palmitic acid-1-C^{14} was injected as before. Radioactivity appeared rapidly in FFA of thoracic duct lymph, and in one of the dogs, in lymph obtained from a channel draining the head and neck (Fig. 3). The appearance of radioactivity in plasma TGFA in these animals was similar to that observed in noncannulated animals. Radioactivity of TGFA in thoracic duct lymph appeared later (Fig. 4). In both animals the specific activity of lymph TGFA exceeded that of plasma TGFA after 45 minutes. In one of the animals triglyceride concentrations in lymph and plasma were similar; in the other the concentration of lymph triglycerides was eight times that of plasma triglycerides (Fig. 4). Practically all the radioactivity in the lymph was contained in the very low density lipoprotein-chylomicron fraction.

A single dog was eviscerated following hepatectomy. Injected palmitic acid-1-C^{14} was removed exponentially, with an initial half life of 3 minutes for 15 minutes, after which the rate slowed as described for intact dogs. Of the total dose injected, 10% appeared in expired CO_2 in 3 hours. No radioactivity was detectable in plasma TGFA in this experiment.

Chylomicron Triglyceride Metabolism. The effect of temporary exclusion of the liver on the rate of removal of chylomicron triglycerides from the circulation was studied in two dogs. In each of these experiments chylomicrons were injected after establishment of the porto-



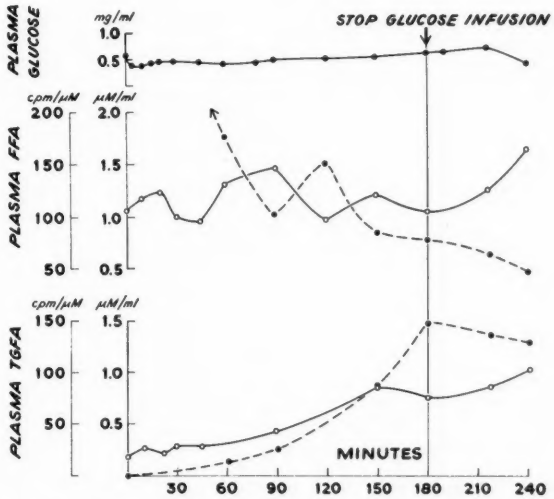


FIG. 2. Concentration of plasma glucose and concentrations and specific activities of plasma FFA and TGFA after intravenous administration of palmitic acid-1-C^{14}, 3.8 × 10^7 cpm, to two hepatectomized dogs. Open circles indicate concentration; closed circles indicate specific activity of FFA and TGFA.

caval shunt and before exclusion of the liver. After a 90-minute interval the liver was excluded from the circulation and a second injection was given. After 30 minutes the liver was returned to the circulation by releasing the ligatures. As shown in Figure 5, in one experiment the rate of removal of the injected triglycerides was slower after the liver was excluded; in the other no significant difference was observed.

Chylomicrons were administered to two hepatectomized and splenectomized dogs. The removal rates were rapid until about 85% of the injected triglycerides had disappeared from the circulation; thereafter the rates slowed markedly (Fig. 6). The specific activity of chylomicron TGFA varied only 10% from the mean of all samples taken in these two experiments. Radioactivity in plasma FFA rose rapidly, reaching a maximum when approximately half the injected triglycerides had been removed from the circulation (Fig. 7). The relative specific activity of the FFA was higher and remained elevated longer in the experiment in which the greater quantity of chylomicron triglycerides had been injected. Specific radioactivity of TGFA contained in lipoproteins which sedimented at density 1.006 did not exceed 5% of that of the injected chylomicrons during the course of these experiments. In one study 1.5% of the radioactivity appeared in expired CO_2 in 2 hours, and in the other, 2.0% appeared in 3 hours. Plasma glucose concentrations were about 110 and 80 mg/100 ml, respectively, and FFA concentrations, 0.3 and 0.5 μmole per milliliter.

Serial samples of omental adipose tissue were excised during these experiments. The lipids were extracted in ethanol-acetone within 15 seconds of removal of the samples. Analyses of the radioactivity of these extracts demonstrated progressive accumulation of C^{14} (Table 1). In the two experiments specific activity of FFA in adipose tissue was much higher than that of TGFA, but much lower than that of FFA in blood plasma obtained concurrently. In both experiments samples of heart, thigh muscle, and small intestine were extracted 2 hours after administration of chylomicrons, and in one, extracts of mesenteric, renal, and subcutaneous adipose tissues were extracted as well. If it is assumed that 5% of body weight in these animals consisted of adipose tissue triglycerides, the percentage of administered C^{14} found in adipose tissue at the end of the experiments can be estimated from the observed specific activities. These values ranged from 30% when the measured specific activity of subcutaneous fat was used in this calculation to 400% when that of mesenteric fat was used. Assuming that skeletal muscle (*a*) had a lipid C^{14} concentration equal to that of thigh muscle, and (*b*) accounts for 40% of body weight, 19% and 27% of the total radioactivity was present in this tissue. The concentration of radioactivity in heart (cpm per gram wet weight tissue) was approximately ten times that of skeletal muscle or intestinal tissue in both studies.

TABLE 1. CONTENT AND SPECIFIC ACTIVITIES OF LIPIDS OF OMENTAL ADIPOSE TISSUE AND BLOOD PLASMA DURING REMOVAL OF CHYLOMICRON TRIGLYCERIDES FROM THE CIRCULATION OF HEPATECTOMIZED DOGS

Minutes After Injection	FFA as Per Cent of TGFA	TGFA	FFA	
		Adipose Tissue	Adipose Tissue	Blood Plasma
		cpm/μmole	*cpm/μmole*	
Exp. 1				
11	0.08	0.8	16	260
24	0.07	1.2	22	300
43	0.06	1.4	7	230
67	0.05	2.2	22	
92	0.05	2.7	43	160
120	0.06	3.6	25	180
Exp. 2				
10	0.07	0.3	4	970
22	0.05	0.6	10	1420
60	0.05	1.3	31	800
120	0.05	1.3	28	260

DISCUSSION

The results of these experiments provide further information concerning the pathways involved in the transport and metabolism of plasma FFA and triglycerides, but they do not permit quantification of the roles of the liver and other tissues under physiologic conditions.

The rate of removal of FFA from the circulation of hepatectomized or eviscerated dogs was about two-thirds that found in intact dogs. Although this observation is compatible with previous evidence that the liver removes a large portion of FFA from the blood, decreased tissue perfusion may have been of equal or greater importance in producing slower removal rates in these experiments. The reduced oxidation of FFA in hepatectomized dogs probably resulted from a variety of factors which cannot be separated, such as decreased cardiac output and altered regional blood flow, absence of the liver, and infusion of glucose. Rapid passage of FFA across the capillary bed in a peripheral area was demonstrated in the experiment in which a cervical lymphatic vessel was cannulated. The mechanism of this rapid transcapillary transport of FFA is not known. The fact that maximal specific activity of lymph FFA

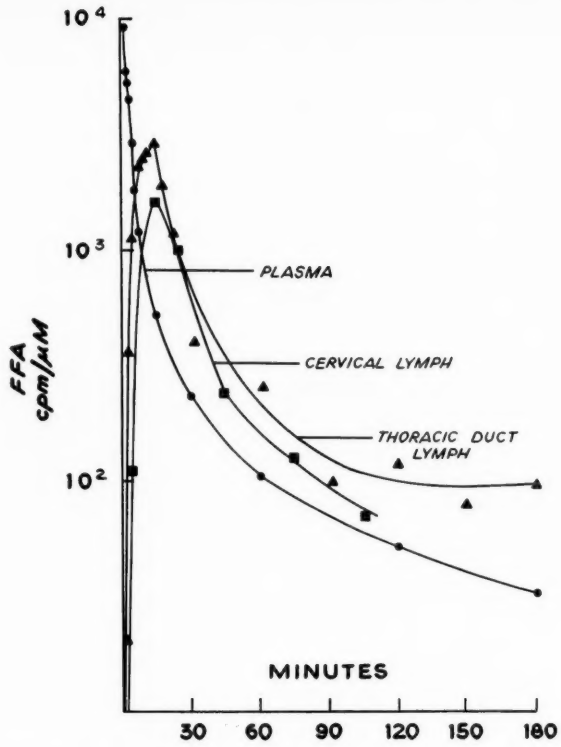


FIG. 3. Specific activities of FFA of plasma and of cervical and thoracic duct lymph after intravenous administration of palmitic acid-1-C^{14} to an intact dog.

was not reached until 15 minutes after injection probably reflects delay in transport from extravascular fluid to the sites of collection.

It is apparent from these studies that the liver is the major site of esterification of plasma FFA to form triglycerides which re-enter the circulation. Laurell (13) recently reached the same conclusion from experiments in rats, in which he compared the specific activities of triglycerides of liver and plasma after injection of palmitic acid-1-C^{14}. Stein and Shapiro (14) perfused rat livers at various intervals after injection of palmitic acid-1-C^{14} and showed that 5% to 20% of the liver triglycerides appeared in the perfusate, in agreement with our studies in intact animals.

Since FFA entering the liver are esterified almost instantaneously,[4] the delay in appearance of labeled triglycerides in the circulation must reflect the time required for transport of triglycerides in liver to the blood. Our studies in dogs with thoracic duct fistulae show that these triglycerides enter the blood stream primarily by way of the hepatic sinusoids rather than through the lymphatic system. Results of our experiments in hepatectomized dogs are similar to those of Harper *et al.* (15), who showed that plasma triglycerides do not become labeled in hepatectomized dogs given C^{14}-labeled acetate. It appears, therefore, that fatty acids synthesized in the liver or FFA delivered to the liver from adipose tissue are sources of plasma triglycerides. It is also likely that triglycerides which enter the liver from chylomicrons can re-enter the plasma. Thus the extent of endogenous triglyceride transport must be a function of hepatic lipogenesis, of the rate of delivery of FFA to the liver from adipose tissue, and possibly of the uptake of chylomicron triglycerides by the liver.

Labeled triglycerides were also found to enter the circulation by way of the thoracic duct. The specific activity of lymph TGFA was found to exceed that of plasma TGFA, even when TGFA concentration was far greater in lymph than in plasma. This observation suggests strongly that some FFA entering intesti-

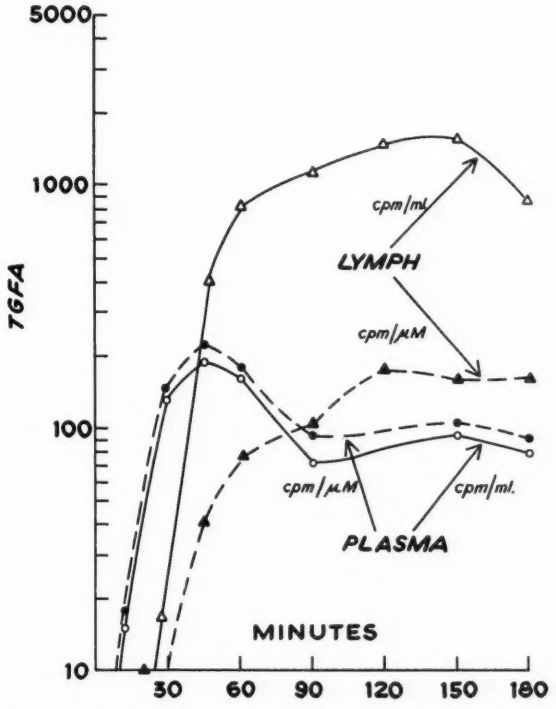


FIG. 4. Radioactivity in TGFA of plasma and thoracic duct lymph after intravenous administration of palmitic acid-1-C^{14}, 3.4×10^7 cpm, to a dog fed cream prior to cannulation of the thoracic duct.

[4] Unpublished data.

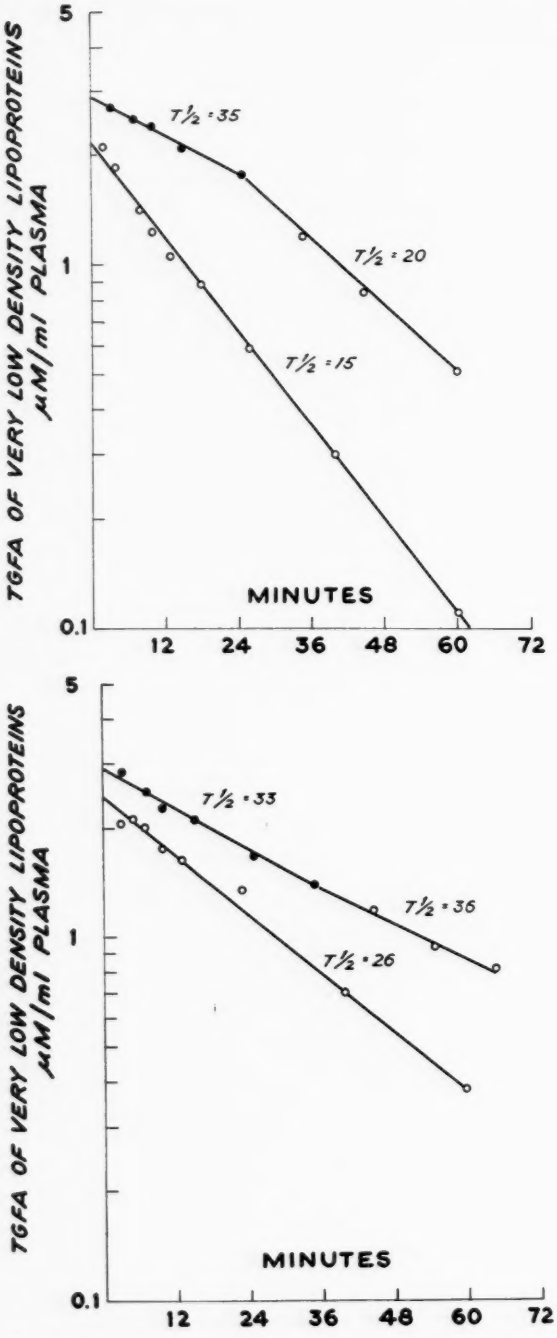


FIG. 5. Disappearance of TGFA from the very low density lipoprotein-chylomicron fraction of plasma after intravenous administration of labeled chylomicrons containing 2.0 mmoles of TGFA to two dogs with portocaval anastomoses. Rate of disappearance of radioactivity from this fraction was practically identical. Open circles indicate hepatic circulation intact; closed circles indicate hepatic circulation occluded.

nal mucosal cells are esterified to form triglycerides which appear in chylomicrons of thoracic duct lymph. Therefore these fatty acids appear to mix, at least in part, with newly absorbed fatty acids at this site, although it is possible that a highly active fraction of plasma lipoprotein triglyceride might pass into the intestinal or hepatic lymph. No evidence was found to support the concept that triglycerides can enter the circulation from adipose tissue as such, although the possibility was not ruled out, since the extent to which the injected palmitic acid-1-C^{14} entered adipose tissue was not determined. Furthermore, it was observed that the concentration of triglycerides failed to fall progressively in hepatectomized animals.

The experiments with labeled chylomicrons demonstrate that the liver is not the only site of removal and oxidation of chylomicron triglycerides, and that hydrolysis of these triglycerides to form circulating FFA occurs in the absence of the liver. This finding is in accord with the reported presence of lipoprotein lipase in

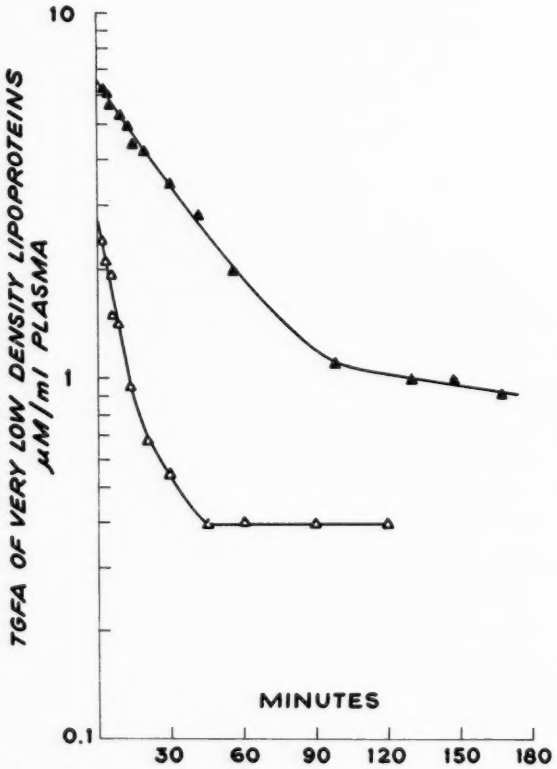


FIG. 6. Disappearance of TGFA from the very low density lipoprotein-chylomicron fraction of plasma of two hepatectomized dogs after intravenous administration of labeled chylomicrons containing 5.8 and 3.6 mmoles of TGFA, respectively.

several peripheral tissues (16). The observation that a large portion of labeled fatty acids derived from chylomicron triglycerides was deposited in adipose tissue is in general agreement with the findings of Bragdon and Gordon in fed rats (2). The large differences in specific activity among different adipose tissues probably reflect several factors, particularly variations in blood flow and enzymatic activities. Since the specific activity of FFA was much lower in adipose tissue than in plasma, it is unlikely that labeled plasma FFA were

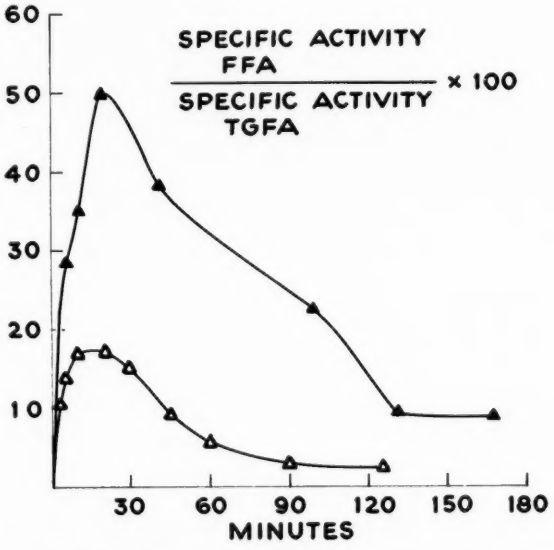


FIG. 7. Specific activities of plasma FFA of two hepatectomized dogs after intravenous administration of labeled chylomicrons. The specific activities are expressed as per cent of that of the TGFA in the administered chylomicrons.

derived from a single pool of FFA in adipose tissue. Although other mechanisms are not excluded, the results suggest that the adipose tissue FFA were derived in part from plasma and in part from adipose tissue triglycerides of very low specific activity. This finding is consistent with the concept (5) that extrahepatic hydrolysis of chylomicron triglycerides occurs prior to entrance of their fatty acids into body tissues, and that localization of fatty acids derived from chylomicron triglycerides in extrahepatic tissues is a function of the local activity of lipoprotein lipase. Such a mechanism is also supported by the recent demonstration by Borgström and Jordan (17) that after injection of chylomicrons labeled in both the glycerol and fatty moieties into rats, the ratio of glycerol to fatty acid radioactivity was much lower in adipose tissue than in plasma.

Our studies raise the question of the fate of circulating triglycerides which are derived from the liver. If their metabolic fate is similar to that of chylomicron triglycerides, they would be expected to enter peripheral tissues containing lipoprotein lipase, and thus provide a mechanism whereby liver triglycerides can be transported to peripheral tissues for utilization or storage. Current studies in rabbits strongly support this concept (18).

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REFERENCES

1. Havel, R. J., and D. S. Fredrickson. *J. Clin. Invest.* **35:** 1025, 1956.
2. Bragdon, J. H., and R. S. Gordon, Jr. *J. Clin. Invest.* **37:** 574, 1958.
3. Gordon, R. S., Jr. *J. Clin. Invest.* **36:** 810, 1957.
4. Roheim, P. S., and J. J. Spitzer. *Am. J. Physiol.* **195:** 288, 1958.
5. Havel, R. J. *Am. J. Clin. Nutrition* **6:** 662, 1958.
6. Dakin, R. L., J. Jew, H. A. Harper, and H. J. McCorkle. *A.M.A. Arch. Surg.* **78:** 856, 1959.
7. Davis, B. D. *Arch. Biochem.* **15:** 351, 1947.
8. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50:** 152, 1957.
9. Borgström, B. *Acta Physiol. Scand.* **25:** 111, 1952.
10. Dole, V. P. *J. Clin. Invest.* **35:** 150, 1956.
11. Carlson, L. A. *Acta Soc. Med. Upsaliensis* **64:** 208, 1959.
12. Fredrickson, D. S., and K. Ono. *J. Lab. Clin. Med.* **51:** 147, 1958.
13. Laurell, S. *Acta Physiol. Scand.* **47:** 218, 1959.
14. Stein, Y., and B. Shapiro. *Am. J. Physiol.* **196:** 1238, 1959.
15. Harper, P. V., Jr., W. B. Neal, Jr. and G. R. Hlavacek. *Metabolism* **2:** 69, 1953.
16. Korn, E. D. In *Methods of Biochemical Analysis*, edited by D. Glick, New York, Interscience Publishers, Inc., 1959, vol. 7, p. 145.
17. Borgström, B., and P. Jordan. *Acta Soc. Med. Upsaliensis* **64:** 185, 1959.
18. Havel, R. J., J. M. Felts and C. M. Van Duyne. *Physiologist* **3:** (No. 3), 76, 1960.

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Release of free fatty acids by adipose tissue *in vivo**

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SUMMARY

A technique is described to determine changes in plasma FFA on passage through adipose tissue *in vivo*. The subcutaneous adipose tissue of the abdominal region was shown to be constantly releasing FFA. After the injection of insulin, mobilization of FFA was depressed in normal as well as in diabetic dogs, but seldom was there any evidence of FFA uptake. Lymph infusion from fat-absorbing donors did not seem to influence the release of FFA by adipose tissue in the recipient animals.

In the past few years considerable effort has been directed to the study of metabolic behavior of adipose tissue. It has been shown that adipose tissue is capable of releasing or taking up fatty acids, depending on the experimental conditions, and that its metabolic activity is influenced by certain hormones *in vitro* (1, 2, 3). While there is little difficulty in obtaining isolated adipose tissue for studies *in vitro*, experiments *in vivo* have been far more limited, and have been restricted largely to sampling of veins which drain, along with adipose tissue, large amounts of muscle or visceral organs (4, 5). The present study is an attempt to provide information concerning the nature of plasma FFA transport through adipose tissue *in vivo* under various conditions. It concerns FFA mobilization in fasting and fat-absorbing animals, as well as the influence of insulin in normal and diabetic animals.

MATERIALS AND METHODS

Mongrel dogs weighing 11 to 24 kgs, and fasted 18 to 20 hours, were used under pentobarbital anesthesia. Arterial and venous samples were obtained through cannulae placed in a side branch of the femoral artery (sample A) and femoral vein (sample V). To obtain samples of blood passing through the subcutaneous adipose tissue of the abdominal region, the inferior epigastric vein was prepared by ligating the vein draining the rectus abdominis muscle, and passing a cannula through the latter vein to its junction with the inferior epigastric vein (sample E). Thus, sample E represented blood coming from the adipose tissue with some contribution from the skin. No muscular drainage could be visibly detected in this sample. Blood flow from the adipose tissue was not interfered with, except during sampling. It was necessary to recannulate this vessel for almost each sampling, to avoid intravascular clotting, since no anticoagulant was administered to the animals in order to preserve physiological conditions as much as possible. Backflow of blood from the femoral vein into the sampled vein was prevented by the valves of the latter vessel, or, if required, by means of a temporary ligature passed around the inferior epigastric vein at its juncture with the femoral vein. In animals in which cannulation of the vein was impossible, samples were obtained by direct venipuncture with a #21 needle. Simultaneous blood samples of an artery, femoral vein, and inferior epigastric vein were withdrawn into syringes at intervals, and immediately transferred to cold, oxalated test tubes and refrigerated at 3°. On each sample, duplicate determinations of FFA were performed by Dole's method (6).

Insulin (0.5 to 1.2 units/kg) was administered intravenously, and arterial glucose changes were followed, using Nelson's colorimetric adaptation of Somogyi's method (7). Seven dogs were made diabetic by an intravenous injection of alloxan (0.75 mg/kg). The dogs were used 48 hours later, after showing marked glycosuria.

* Supported by grants from the National Institutes of Health.

The effect of fat absorption was studied by employing two dogs for each of three experiments. The donor was fed 20 ml triolein, and the recipient was simultaneously anesthetized. Control samples from the recipient animal were taken several hours later, and shortly before infusion of chyle. The donor dog was anesthetized 3 hours after feeding, and its thoracic duct was cannulated. The distal end of this cannula was inserted into the jugular vein of the recipient dog, and chyle was permitted to flow in this manner for 90 minutes or more, with blood samples being drawn from the recipient dog at intervals. FFA, glucose, and esterified fatty acids were determined in the plasma samples.

TABLE 1. STUDIES ON NORMAL DOGS

Dog and Sample No.	Time	Sample A	Sample V	Sample E	E − A
	min				
M 2—1	0	0.673	0.635	0.726	0.053
2	20	0.715	0.811	0.781	0.066
M 3—1	0	0.536	0.963	0.899	0.363
2	10	0.564	1.065	0.959	0.395
3	20	0.565	0.788	0.886	0.321
4	35	0.556		0.802	0.246
5	45	0.504	0.848	0.788	0.284
M 4—1	0	0.538	0.610	0.574	0.036
2	15	0.558	0.611	0.482	−0.076
3	45	0.445	0.542	0.438	−0.007
F 5—1	0	0.636	1.170	0.896	0.260
2	20	0.860	1.300	1.200	0.340
3	50	0.706	1.480	0.966	0.260
F 6—1	0	0.720	0.966	1.277	0.557
M 7—1	0	0.377	0.556	0.691	0.314
M 8—1	0	0.676	0.720	0.919	0.243
M 9—1	0	1.266	1.369	1.556	0.290
M 10—1	0	1.380	1.625	1.690	0.310
F 11—1	0	0.331	0.429	0.430	0.099
2	15	0.340	0.425	0.462	0.112
M 12—1	0	0.230	0.258	0.328	0.098
2	12	0.223	0.213	0.297	0.074
M 13—1	0	0.763	0.830	0.950	0.187
2	10	0.798	0.888	0.910	0.112
3	32	0.936	0.975	1.044	0.108
M 14—1	0	0.316	0.350	0.386	0.070
2	10	0.282	0.319	0.365	0.083
F 20—1	0	0.379	0.493	0.937	0.558
2	5	0.365	0.538	1.029	0.664
3	100	0.341	0.378	0.974	0.633
F 24—1	0	0.756	0.900	0.896	0.140
2	40	0.589	0.695	0.718	0.129
3	82	0.579	0.694	0.696	0.118
M 25—1	0	0.264	0.332	0.596	0.332
2	3	0.279	0.332	0.550	0.271
F 27—1	0	0.281	0.232	0.293	0.012
2	105	0.179	0.254	0.244	0.065

All FFA values in mEq/liter.

It should be noted that dog #20 had milk-laden *post partum* mammary glands, and these tissues were drained by the inferior epigastric vein.

RESULTS

Table 1 shows a quite consistent release of FFA by the adipose tissue, i.e., higher concentrations in the epigastric vein than in the artery. The magnitude of this release equals about 43% of the arterial concentration (calculation based on the first sample of each animal).

After administration of insulin, the arterial FFA level often decreased within 10 minutes after injection, and frequently rather markedly. The average decrease in epigastric vein−arterial (E−A) FFA difference after insulin was 0.179 mEq/liter, with a standard error of 0.05. In six out of the eight dogs the E−A difference decreased within 25 minutes after injection. In the two remaining dogs there was no apparent cessation or near cessation of FFA release from the subcutaneous adipose tissue at some time within 25 minutes after insulin, and in one of these, a negative E−A difference was observed for the same tissue. In about half of the experiments an uptake of glucose by the adipose tissue was found after the administration of insulin. One typical experiment is shown in Figure 1.

As expected (8), the diabetic dogs had, statistically, significantly higher arterial FFA concentrations (mean = 1.116) than the normal dogs used in this study (mean = 0.566). In all samples but one the epigastric vein

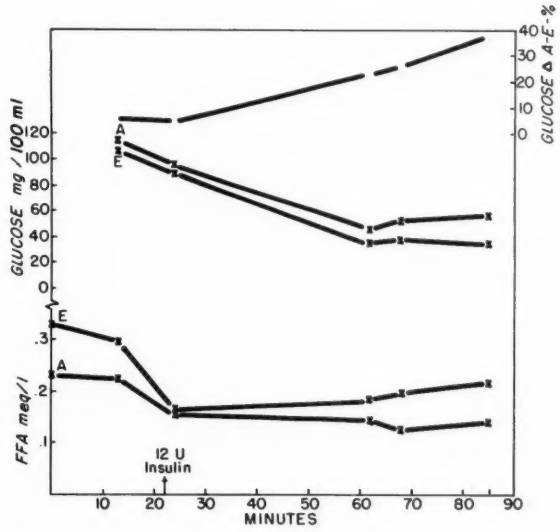

FIG. 1. Effect of insulin injection on FFA release in a normal dog.

had higher FFA concentration than the artery, the differences being similar to those found in normal animals.

In all cases E−A differences were decreased after insulin. One typical experiment is shown in Figure 2. The average decrease in E−A difference was found to be 0.121 meq/liter, with a standard error of 0.028. In two dogs marked uptake of FFA by adipose tissue was observed after insulin. In one of them the uptake was present even before insulin.

E−A differences did not differ markedly during lymph infusion, although a slight increase was noticed. One example of these studies is given in Figure 3.

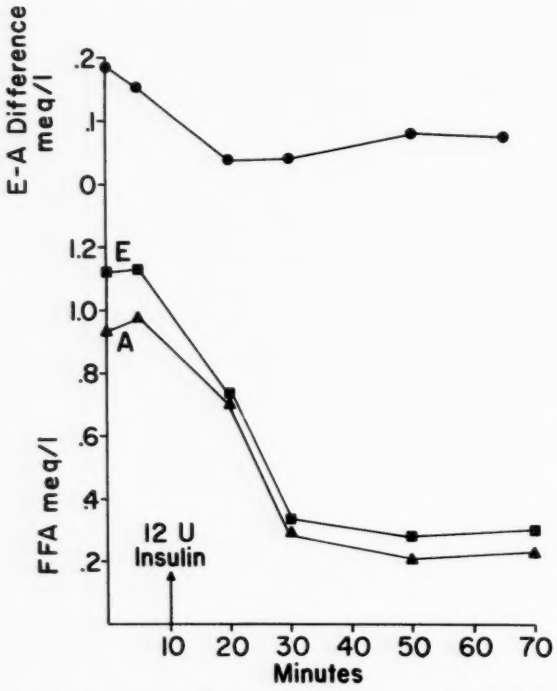

FIG. 2. Effect of insulin injection on FFA release in a diabetic dog.

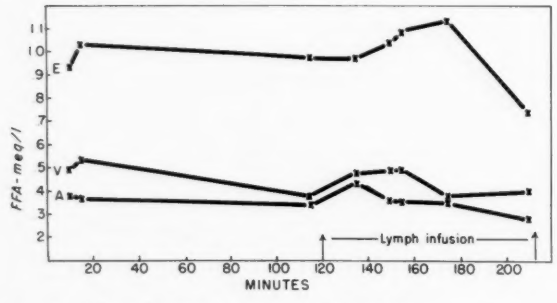

FIG. 3. Effect of lymph infusion on FFA release.

DISCUSSION

Simultaneous determinations of E−A FFA differences yielded valuable information on the behavior of adipose tissue, but this information cannot be translated into strict quantitative terms, unless the amount of blood flow through the adipose tissue in question is known. Our attempts to determine this have been unsuccessful so far. Therefore the reported changes can represent quantitative information only if one assumes that no major change in blood flow through adipose tissue occurred in the course of the experiments. Such assumption is justified under the present experimental conditions since neither diabetes nor the same amount of insulin seems to influence blood flow through coronary arteries, liver, or thigh muscles.[1] Even excessive doses of insulin (40 to 65 units/kg) caused only a small (10% to 20%) increase in femoral venous flow after about 10 minutes in dogs (9). In man (10), massive doses of from 40 to 280 units of insulin increased blood flow in the hand and forearm in 60 to 145 minutes after injection. The relatively small volume of infused lymph was also unlikely to alter blood flow through adipose tissue. In the normal animal a continuous release of FFA could be observed from the adipose tissue. Hormonal influences undoubtedly play a role in influencing this release. Insulin decreased it; epinephrine seemed to increase it. The latter hormone was tested only in one experiment, because any change caused by epinephrine is hard to interpret, depending on the simultaneous blood flow changes that occur. Although the arterial FFA level has an influence on the uptake of FFA by both the liver (11, 12) and the myocardium (13), it does not seem to correlate with the release of FFA by adipose tissue.

Using the Fick principle, rapidly falling arterial concentration of the measured metabolite may introduce an error. Since an E−A FFA gradient existed in the present experiments, such error would have increased rather than decreased the E−A difference. Thus the decreased E−A difference across the adipose tissue after insulin administration cannot be explained on this basis.

It is difficult either to prove or disprove a cause-effect relationship from the present data between the diminished E−A difference and the lowering of arterial FFA after insulin. Seldom was there any evidence for FFA uptake by adipose tissue caused by insulin.

The effect of insulin on FFA release from the adipose tissue *in vivo* is similar to its effect in the presence of glucose *in vitro* (14). The uptake of glucose by the adipose tissue after insulin that occurred in half of the

[1] Unpublished observation.

experiments is also reminiscent of the effect of insulin on adipose tissue *in vitro* (2).

Adipose tissue continued to release FFA durmg lymph infusion, in spite of the greater influx of triglycerides and of FFA (15) from the lymph to the blood.

It can be observed from Table 1 that the FFA level in the femoral vein is frequently equal to or higher than that in the epigastric vein. Since only a portion of the femoral venous blood comes from adipose tissue, one possibility is that deep-lying adipose tissue is perhaps more active, metabolically, than the subcutaneous tissue. Difference in temperature may be one of the possible explanations. Similar assumptions could be made from experiments involving the region (predominantly muscles) drained by the profunda femoris vein in the dog; this vein frequently contains higher concentration of FFA than the femoral vein (16). Therefore more information is needed before the findings of the present study can be safely applied to all adipose tissue regardless of location.

The valuable help of Dr. H. I. Perlmutter in working out the details of the anatomy of the epigastric vein is gratefully acknowledged.

REFERENCES

1. Gordon, R. S., Jr., and A. Cherkes. *Proc. Soc. Exptl. Biol. Med.* **97**: 150, 1958.
2. Leboeuf, B., R. B. Flinn and G. F. Cahill, Jr. *Proc. Soc. Exptl. Biol. Med.* **102**: 527, 1959.
3. Reshef, L., and B. Shapiro. *Metabolism, Clin. and Exptl.* **9**: 551, 1960.
4. Gordon, R. S., Jr. *J. Clin. Invest.* **36**: 810, 1957.
5. Roheim, P. S., and J. J. Spitzer. *Am. J. Physiol.* **195**: 288, 1958.
6. Dole, V. P. *J. Clin. Invest.* **35**: 150, 1956.
7. Nelson, N. *J. Biol. Chem.* **153**: 375, 1944.
8. Bierman, E. L., V. P. Dole and T. N. Roberts. *Diabetes* **6**: 475, 1957.
9. Ludwigs, N., and W. Rossel. *Arch. Physiol. Pfluger's* **257**: 137, 1953.
10. Abramson, D. I., N. Schkloven, M. N. Margolis, and I. A. Mirsky. *Am. J. Physiol.* **128**: 124, 1940.
11. McElroy, W. T., Jr., W. L. Siefert and J. J. Spitzer. *Proc. Soc. Exptl. Biol. Med.* **104**: 20, 1960.
12. Spitzer, J. J., and W. T. McElroy, Jr. *Am. J. Physiol.* **199**: 876, 1960.
13. Finkelstein, L. J., J. J. Spitzer and J. C. Scott. *Circulation* **22**: 679, 1960.
14. Engel, F. L., and J. E. White, Jr. *Am. J. Clin. Nutrition* **8**: 691, 1960.
15. Spitzer, J. J. *The Blood Lipids and Clearing Factor.* Brussels, Royal Flemish Academy, 1956, p. 243.
16. Issekutz, B., Jr., and J. J. Spitzer. *Proc. Soc. Exptl. Biol. Med.* **105**: 21, 1960.

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Effect of ingested fat on fatty acid composition of serum lipoproteins

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SUMMARY

The fatty acid composition of several serum lipoprotein fractions of a human subject was studied before and after the ingestion of a fat meal composed of corn oil. It was found that the fatty acids of the chylomicrons, low density lipoproteins ($d < 1.019$), and of the $d > 1.21$ fraction tend to resemble the fatty acids of the recently ingested fat. It was established that this resemblance was not the result of exchange *in vitro* from chylomicrons to other fractions. Similar changes in composition of the other lipoprotein fractions were also observed, but these were small in magnitude and not outside the range of experimental error.

This investigation was undertaken to determine the effect of recently ingested fat on the fatty acid composition of several serum lipoprotein fractions. It had been previously demonstrated that following the ingestion of different fats, the fatty acid composition of chylomicrons of both rat and man resembles the composition of the fat fed (1). This finding has been confirmed in human chyle (2), in which it also appeared that the fatty acid composition of other lipoproteins underwent similar, but less definite, changes. A second purpose of the present study was to determine what part exchange of fatty acids between chylomicrons and various lipoproteins *in vitro* might play in these observations.

METHODS

The subject was a healthy 46-year-old male technician. No attempt was made to control his usual diet, except that he was requested to eat a relatively low fat meal the evening before the experiment. A blood sample was taken, in the fasting state, at about 9 A.M. He then drank 100 g of corn oil emulsified in skim milk. He was bled again 6 hours later. The blood was permitted to clot and the serum was removed. The fasting serum sample was clear; the second sample was very lipemic. The same experiment was repeated on the same subject 15 days later.

Corn oil was selected for these experiments because of its relatively high concentration of linoleic acid compared to that of most serum lipids. Since oleic acid was found in substantial concentration in each of the lipoproteins, an increase in the linoleic to oleic ratio of these two acids, rather than the total fatty acid composition, is presented.

The chylomicrons were separated from the serum by layering 7 ml of the latter under a phosphate-citrate buffer (pH 7.2, 0.05 M, density 1.006) in lusteroid tubes with a total volume of about 13.5 ml. Four tubes of each serum sample were so prepared. They were centrifuged for 30 minutes at 20,000 rpm at room temperature in the 40 rotor of the Spinco preparative ultracentrifuge. The chylomicrons were thereby concentrated in the top few milliliters of the tube, from which they were removed after slicing the tube. The chylomicrons were washed by centrifuging three times through the buffer under conditions similar to the original isolation, except that no attempt was made to layer them. In the case of the fasting sample, the chylomicrons from the original four tubes were progressively pooled, so that the final wash was accomplished in one tube only.

In order to diminish the possibility that some chylomicrons might remain in the serum, the infranatant material from the first centrifugation was again layered under buffer, centrifuged under the same conditions, and the tops (slightly lactescent in the case of the second sample) were discarded.

The serum, thus twice cleared of chylomicrons, was then fractionated by repeated centrifugations at increasing densities by the method of Havel *et al.* (3). The fractions were extracted in chloroform-methanol and the extracts washed with slightly acid water. Suitable portions of the chloroform phase were evaporated, and methyl esters of the fatty acids were prepared by incubation of the dried lipid with 1-ml aliquots of 98% methanol and 2% sulfuric acid for 16 hours at 65° in sealed test tubes. Water (5 ml) was then added to each tube and the lipids were extracted into petroleum ether. The petroleum ether extract was carefully blown to dryness with a stream of nitrogen; the lipids were taken up in isooctane; and aliquots of these solutions were analyzed by gas-liquid chromatography. The columns used were 6-foot glass columns filled with Chromosorb W coated with a quantity of ethylene glycol adipate polyester approximately 10% of the weight of the Chromosorb. The columns were run at 200° and the gas flow was 100 ml/minute. A modified argon ionization detector (4), which had been calibrated for quantitative accuracy within a specific range of vapor concentration, was used, and the size of the aliquots analyzed was experimentally adjusted so that no component of the mixture gave a vapor concentration during chromatography which exceeded that range. Quantification was accomplished by measuring the areas under the curves.

RESULTS

The results are presented in Table 1, in which the ratio between the concentrations of linoleic and oleic acids are given for the several fractions. It is apparent from the fasting samples that this ratio increased in all fractions during the interval between the two experiments. Six hours following the ingestion of the corn oil, in both experiments, there was an increase in this ratio in all fractions. The increase was greatest in the chylomicrons. The ratio approximately doubled in the fraction of density <1.019, and in the fraction of density >1.21. The changes in the other fractions were smaller.

TABLE 1. RATIO OF LINOLEIC TO OLEIC ACIDS IN SEVERAL SERUM LIPOPROTEIN FRACTIONS BEFORE AND AFTER FEEDING CORN OIL*

	Experiment 1		Experiment 2	
	Before	After	Before	After
Chylomicrons	0.37	1.81	0.48	1.82
1.019 top	0.44	0.95	0.55	1.04
1.063 top	1.08	1.32	1.33	1.44
1.21 top	1.01	1.13	1.24	1.59
1.21 bottom	0.49	1.41	0.77	1.33

* This ratio in the corn oil fatty acids was 2.28.

To assess the reliability of the methods, and to ascertain if an appreciable exchange of fatty acids between chylomicrons and other lipoproteins occurred, the fasting serum in the first experiment, after twice centrifuging off the chylomicrons, was treated in the following way: Five milliliters of serum was placed in each of three test tubes; 1.5 ml of buffer was added to the first; and 1.5 ml of washed chylomicrons from the postprandial sample was added to each of the other two tubes. In tube 2 the chylomicrons were added at the end of the incubation, and in tube 3 they were added at the beginning of the incubation. Incubation took place at room temperature for 2 hours. Lipid (8.2 mg) was added as chylomicrons to 5 ml of serum containing 22.5 mg of lipid. It should be realized that the lipid content of human serum chylomicrons is about 90% triglyceride, whereas in fasting serum the triglycerides constitute only about 15% of the total lipid.

The contents of these three tubes were then fractionated and analyzed exactly as described above. The results are shown in Table 2. The greatest differences occur between tubes 1 and 2, i.e., between the control without added chylomicrons and the zero-time control. In most fractions the zero-time sample actually shows a lower linoleic to oleic ratio than the control. We believe that these differences demonstrate the range of experimental error. A comparison of tube 3 with either tube 1 or tube 2 demonstrates that no significant exchange of fatty acids from chylomicrons to other lipoproteins occurs *in vitro*.

TABLE 2. RATIO OF LINOLEIC TO OLEIC ACIDS IN SEVERAL SERUM LIPOPROTEIN FRACTIONS AFTER INCUBATING FASTING SERUM WITH CORN-OIL CHYLOMICRONS

	Serum and Buffer	Serum and Chylomicrons	
	Tube 1	Tube 2	Tube 3
	2 hours	*zero time*	*2 hours*
Chylomicrons		1.75	1.79
1.019 top	0.44	0.39	0.47
1.063 top	1.08	0.78	1.16
1.21 top	1.01	0.82	0.96
1.21 bottom	0.49	0.57	0.60

The approximate doubling of the linoleic to oleic ratio in the 1.019 top and 1.21 bottom fractions, as seen in Table 1, appears to be greater than one might expect from experimental error.

DISCUSSION

We have no explanation for the increased linoleic to oleic ratio in the fractions of fasting serum in the interval between experiments. One might conclude that such observations without rigid prior control of diet have little significance.

The fatty acids in the 1.21 bottom fraction represent primarily the free fatty acids (FFA) of the serum, although from the data of Phillips (5), it would appear that about 300 μEq/liter of fatty acids in this fraction are present as phosphatides, primarily lysolecithin. It was reported by Dole (6) that the chemical composition of the FFA of the serum could not be significantly altered by short-term changes in the type of fat fed. We accepted these data and in a previous paper (1) interpreted them as indicating that the FFA were in such rapid exchange with the depot fats that chemical equilibrium with the latter would be a constant feature. It has been shown by several groups of workers (7) that the half life of intravenously injected radioactively labeled FFA is a matter of only 1 or 2 minutes. It has also been shown that the feeding of a fatty meal results in only slight increases in levels of FFA. But the data presented here, although limited in extent, indicate that the composition of the FFA of the serum can presumably be influenced by the composition of fat recently ingested.

The doubling in the ratio linoleic to oleic in the 1.019 top fraction after feeding corn oil (Table 1) could be explained in several ways. Some fat may be absorbed from the intestine in the form of these very low density lipoproteins. Chylomicrons certainly vary greatly in size, and presumably include a spectrum just as do the low density lipoproteins. The problems of defining, and indeed of separating, these spectra have not yet been resolved. It can be pointed out in this regard, however, that the 1.019 top fractions in these two experiments showed only minimal lactescence. In experiment 1 the amount of total lipid in these fractions was determined chemically. The postprandial sample contained less than a 10% increment over the fasting sample.

Another possible explanation is that the chylomicrons are degraded *in vivo*, by the enzymatic removal of triglyceride, into low density lipoproteins. This opinion has been expressed by Lindgren *et al.* (8). We tend to favor the view that these very low density lipoproteins have a relatively short half life (a matter of hours), and that their fatty acid composition reflects that of the fat recently transported to the liver, where they presumably arise (9).

The increase in linoleic to oleic ratio in the other lipoprotein fractions (1.063 top and 1.21 top) after feeding corn oil, although not significant, is consistent. It should be recalled, in this respect, that only a small percentage of their constituent lipid is triglyceride. The increased ratio may represent binding of FFA (10).

REFERENCES

1. Bragdon, J. H., and A. Karmen. *J. Lipid Research* **1:** 167, 1960.
2. Kayden, H. J., A. Karmen, A. E. Dumont, and J. H. Bragdon. *J. Clin. Invest.* **39:** 1001, 1960.
3. Havel, R. J., H. A. Eder and J. H. Bragdon. *J. Clin. Invest.* **34:** 1345, 1955.
4. Lovelock, J. E. *J. Chromatog.* **1:** 34, 1958.
5. Phillips, G. B. *Proc. Soc. Exptl. Biol. Med.* **100:** 19, 1959.
6. Dole, V. P., A. T. James, J. P. W. Webb, M. A. Rizack, and M. F. Sturman. *J. Clin. Invest.* **38:** 1544, 1959.
7. Fredrickson, D. S., and R. S. Gordon, Jr. *Physiol. Revs.* **38:** 585, 1958.
8. F. T. Lindgren, N. K. Freeman, A. V. Nichols, and J. W. Gofman. In *The Blood Lipids and the Clearing Factor*, 3rd International Conference on Biochemical Problems of Lipids, Brussels, 1956, p. 224.
9. Radding, C. M., and D. Steinberg. *J. Clin. Invest.* **39:** 1560, 1960.
10. Shafrir, E. *J. Clin. Invest.* **37:** 1775, 1958.

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Evidence for the physiological occurrence of lysolecithin in rat plasma*

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SUMMARY

An improved separation of phospholipids on activated silicic-acid columns was achieved by stepwise elution with increasing concentrations of methanol in chloroform. Chemical analyses and paper strip chromatography demonstrated that the noncholine-containing fraction was free of choline-containing phospholipids, and that the lecithin and lysolecithin were of high purity. Rat plasma was shown to contain on the average 17.5% of its lipid phosphorus in the form of lysolecithin. The following is evidence that plasma lysolecithin is not an *in vitro* breakdown product: (*a*) rapid glass-paper chromatography of lipid extracts showed the presence of a distinct spot with the R_f of lysolecithin, (*b*) after P^{32} injection the specific activity of lysolecithin was less than that of lecithin, (*c*) silicic-acid chromatography of C^{14}-labeled plasma lipids failed to show a significant liberation of fatty acids, and (*d*) rechromatography of P^{32}-labeled lecithin did not give rise to a lysolecithin peak.

While lysolecithin has been demonstrated in solvent extracts of human plasma (1 to 5), thus far no evidence has been presented that lysolecithin is present in the blood of intact animals. It would therefore appear legitimate to inquire whether the lysolecithin in lipid extracts might be a degradation product produced during extraction, purification, or chromatographic analysis. In the present paper evidence is presented for the existence of lysolecithin in the blood of intact rats.

Published methods for the separation of plasma phospholipids either failed to detect lysolecithin (6, 7, 8), or the lecithin and lysolecithin (2, 3) fractions were not very pure. Combination of the small silicic-acid column of Phillips (2) with the approximate solvent proportions proposed by Hanahan *et al.* (9) allowed the isolation of these two phospholipids from rat plasma with sufficient purity for our measurements.

METHODS

Normal Holtzman male rats weighing 500 to 600 g were maintained on Purina laboratory chow ad libitum. Large rats were purposely employed so that sufficient plasma phospholipids could be obtained from individual animals for the isotopic studies. In several experiments rats received a single injection of inorganic P^{32} intraperitoneally. At 6, 16, 18, and 20 hours after P^{32} injection, blood samples were taken from the dorsal aorta under Na-pentobarbital (30 mg/kg) anesthesia. Two per cent Mepesulfate (Hoffmann-LaRoche, Inc.) in 0.9% saline was used as an anticoagulant unless otherwise stated. Blood samples were placed in ice water and immediately centrifuged at 1,000 × *g* for 10 minutes at 4°. The supernatant plasma was extracted at 4° overnight with chloroform-methanol 2:1 (v/v)[1] and then purified according to the method of Folch *et al.* (10), except that two gentle

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† Career Investigator of the American Heart Association.

[1] The chloroform used in all procedures was freshly distilled technical grade containing 0.25% amylene as preservative. The methanol used in all procedures, except where noted, was freshly distilled technical grade found to contain 3% water.

inversions with equal volumes of distilled water were used for washing. The washed extract was taken to dryness *in vacuo* at room temperature in a rotary flash evaporator and the residue redissolved in 10 ml of chloroform.

Column Separation of Phospholipid Fractions. Four grams of Mallinckrodt 100-mesh silicic acid (suitable for chromatographic analysis), freshly activated[2] by heating at 120° to 130° for 48 hours, was slurried with chloroform and transferred to a 10 × 100-mm column with a 250-ml solvent reservoir. The silicic acid was packed with sufficient pressure from dried air (approximately 1 psi) to maintain a flow rate of 0.5 ml/minute. The phospholipid sample in about 10 ml of chloroform was added, and the pressure increased gradually to 4 to 5 psi to maintain the same flow rate. The eluates from the column were automatically collected through a volumetric siphon in 5-ml fractions.[3] Loads of up to 1.4 mg of phospholipid phosphorus have been employed with satisfactory results.

When the sample had completely entered the column, it was washed with an additional 30 ml of chloroform, which eluted most of the neutral lipids. Approximately 40 to 50 ml of 20% methanol in chloroform (v/v) was then added to the column to elute the noncholine-containing phospholipids. The choline-containing phospholipids, which were left on the column, were separated by adding a series of solvents as follows: 125 to 140 ml of 40% methanol in chloroform (v/v) eluted lecithin; 50 to 80 ml of 60% methanol in chloroform (v/v) eluted sphingomyelin; finally, 50 ml of methanol eluted lysolecithin.

The amount of water in the methanol employed for the eluant mixture was found to be critical. The methanol we employed contained 3% water. The substitution of absolute methanol, prepared by refluxing with magnesium and a trace of iodine, gave poor separations. Distilled technical or reagent grade chloroform could be used interchangeably in the solvent mixture. Other factors that had to be controlled were the particle size and the condition of the silicic acid. Use of the silicic acid prepared by the method of Hirsch and Ahrens (7) (Bio-Rad 325 mesh), either in the form shipped or activated according to our conditions, gave far poorer separations than did the original Mallinckrodt product. Nonactivated Mallinckrodt silicic acid also gave poor separations of phospholipid classes. Furthermore, silicic acid, activated and kept in a desiccator over sulfuric acid, gradually lost its ability to separate phospholipid mixtures.

Aliquots from each 5-ml fraction were analyzed for phosphorus by the method of Bartlett (11), modified as follows: the aliquots were evaporated to dryness in a water bath, and then to each sample was added 0.2 ml of concentrated sulfuric acid. This mixture was digested in a heating block at 210° for 30 minutes. Three to four drops of 30% phosphorus-free hydrogen peroxide (Mallinckrodt) then was added to each tube, and the tubes were again heated at the same temperature for another 30 minutes in order to decompose all of the oxidizing agent. Solvent extracts were mounted and counted on aluminum planchets (12).

Separation of C^{14}-Labeled FFA from Phospholipids. C^{14}-labeled fatty acids were prepared from the liver of a rat which had previously received 200 μc of C^{14}-acetate. Hydrolysis of the extracted lipids was carried out with 0.2 M NaOH in the presence of carbon tetrachloride-methanol-water at 60° for 30 minutes according to Dawson (13). After removal of nonsaponifiable lipids, fatty acids were extracted with petroleum ether (Skellysolve B, b.p. 60°–70°). The residue of C^{14}-fatty acids, after solvent evaporation *in vacuo*, was dissolved in chloroform and added to a chloroform extract of neutral lipids and phospholipids of rat plasma, respectively. Both samples were then placed on a silicic acid-Super-Cel 1:1, (w/w) column (10 × 40 mm). The silicic acid (Mallinckrodt) had been previously equilibrated with saturated $CaCl_2$ solution which provided a relative humidity of 30% at room temperature.[4] Neutral lipids and fatty acids were eluted with 20 ml of chloroform, and phospholipids with 20 ml of methanol. In both samples recovery of C^{14}-fatty acids in the chloroform eluate was better than 90%. It is interesting to note that a petroleum ether extract of phospholipid fatty acids, when evaporated at atmospheric pressure at 60° to 70°, furnished fatty acids that could no longer be recovered quantitatively from the silicic-acid column with chloroform or methanol.

Determination of Phospholipid Glycerol. Glycerol was determined by a modification of the method of Van Handel and Zilversmit (14). Chromotropic acid was prepared by dissolving 110 mg of disodium 4,5-dihydroxy 2,7-naphthalene disulfonate (Eastman) in 10 ml of distilled water. To reduce absorbence of the reagent blank, the solution was filtered through What-

[2] Henceforth, this silicic acid will be designated as activated silicic acid. Fractions collected from this column without a sample load showed the absence of phosphorus and the presence of negligible amounts of glycerol-like material (i.e., formaldehyde after periodate oxidation).

[3] In several instances the separation was monitored by running the eluate through a polyethylene tube (PE 160) wound around a 1B85 Victoreen Geiger tube and measuring the P^{32} radioactivity through a rate meter and recorder.

[4] Henceforth, this silicic acid will be designated as nonactivated silicic acid.

man No. 1 filter paper. To the filtrate was added 90 ml of 24 N H_2SO_4, and the mixture was stored in a dark bottle at 4°.

A sample containing up to 100 μg of lipid phosphorus was dried in an Erlenmeyer flask and resuspended in 1 ml of absolute ethanol. Five milliliters of 2 N HCl was added and the sample refluxed for 48 hours. The entire sample was then shaken with 5 ml of distilled Skellysolve F, b.p. 30°–40°, as in the procedure of Carlson and Wadström (15), and filtered through Whatman No. 31 filter paper. To 0.2 ml of the filtrate was added 0.1 ml of 0.01 M KIO_4. After mixing and a 10-minute waiting period, 0.2 ml of 5% $NaHSO_3$ was added. Then 3 ml of chromotropic acid was added and the contents again mixed and heated at 100° for 30 minutes in the absence of light. After the heating period the sample was cooled, 0.2 ml of 10% aqueous thiourea was added, and the contents again mixed. Thiourea was added to lower the reagent blank according to Frisell and Mackenzie (16). The resulting color was read at 570 mμ against 4 μg glycerol standards. To determine the relative amounts of free and phosphorylated glycerol, we analyzed for phosphate on digested and undigested samples.[5] In one instance the total phosphate of the lecithin hydrolyzate was 9.7 μmoles, whereas the inorganic phosphate was 4.6 μmoles. By difference, the glycerophosphate was therefore 5.1 μmoles or 0.53 of the total phosphate. Upon reaction with periodate, one obtains for every mole of glycerol 2 moles of formaldehyde, and for every mole of α glycerolphosphate only one mole of formaldehyde. To obtain the total μmoles of glycerol of the lecithin hydrolyzate, one multiplies the μmoles of formaldehyde by an appropriate factor, in this instance 0.76 [0.47/2 + 0.53].

Preparation of Lecithin and Lysolecithin Standards. Dog bile was extracted and washed according to the method of Folch *et al.* (10), and then phospholipids were separated from neutral lipids on a silicic-acid column. One gram of nonactivated silicic acid-Super-Cel 1:1 (w/w) was slurried in chloroform and transferred to a 10-ml syringe barrel fitted with a glass wool plug. The sample, in chloroform, containing 0.75 mg of lipid phosphorus, was placed on the column, and the neutral lipids were eluted with 20 ml of chloroform. The phospholipid was eluted with 20 ml of methanol. A small amount of silicic acid in the eluate was removed by centrifugation prior to solvent evaporation at room temperature *in vacuo*. The phospholipid, which by column and paper chromatography was shown to be predominantly lecithin, was taken up in peroxide-free diethyl ether and incubated with lyophilized *Crotalus adamanteus* snake venom (Ross Allen's Reptile Institute, Silver Springs, Fla.) to prepare lysolecithin according to the method of Long and Penny (18). Both lecithin and lysolecithin served as standards for paper chromatography.

Other Methods. Characterization of phospholipids by glass paper chromatography was performed by the methods of Brown *et al.* (19) and Dieckert *et al.* (20). All R_f measurements were made from the origin to the center of the spot. Choline-containing phospholipid spots were detected by the Reinecke salt method, and the detection of amino groups was made by the ninhydrin reaction (21). Ester bonds were determined by the method of Rapport and Alonzo (22), as modified by Hirsch and Ahrens (7), who substituted *n*-butyl alcohol for the ethanol employed as the solvent for the ferric perchlorate. Methyl palmitate (California Corporation for Biochemical Research) was used as a standard. Choline was determined by the method of Wheeldon and Collins (23), but the samples were read at 715 mμ, which increased the sensitivity by 10%. Sphingomyelin was differentiated from other phospholipids by the process of mild alkaline hydrolysis (24). The determination of phosphorus in the water phase was found difficult, however, because it involved the evaporation of large volumes of trichloroacetic acid solution for acid digestion. In addition, the salt which formed during the process of saponification made the solution turbid. To eliminate this defect, the samples were evaporated in Erlenmeyer flasks and digested with 1 ml of concentrated sulfuric acid on a hot plate at 210° for 30 minutes. The volumes of other required reagents were proportionally increased. After the color developed, the sample was transferred to a centrifuge tube, cooled in ice, and extracted by shaking and subsequent centrifugation with known volumes of isobutyl alcohol (25). Finally, a small amount of crystalline sodium chloride was added to accelerate the process of the extraction of color. The isobutanol extract was read at 780 mμ.

RESULTS

Characterization of Phospholipid Fractions. The phospholipid fractions of normal rat plasma were found to be separable into four fractions: noncholine, lecithin, sphingomyelin, and lysolecithin (Fig. 1). Lipid phosphorus eluted from 20 silicic-acid columns

[5] Undigested α- or β-glycerolphosphate were found to give no color with the Fisk SubbaRow reagent. There is the possibility that the estimation of free glycerol from periodate oxidation might be in error due to the presence of β-glycerolphosphate, which does not oxidize to formaldehyde. However, at low pH, as in the conditions of our hydrolysis, the equilibrium strongly favors the α form (17).

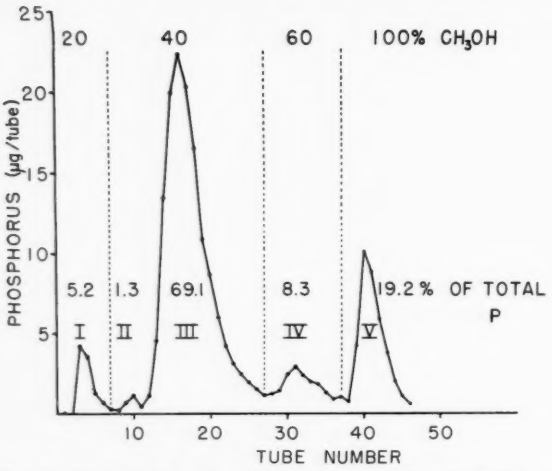


FIG. 1. Representative chromatographic separation of rat plasma phospholipids. The peaks represent: *I* and *II:* noncholine-containing; *III:* lecithin; *IV:* sphingomyelin; *V:* lysolecithin. Phospholipid load: 201 μg P.

was recovered to the extent of 99.1 ± 1.6 S.D. per cent of the amount applied to the column. The amount of lipid phosphorus from each peak was expressed as a percentage of the total lipid phosphorus applied to the column. These percentages were fairly reproducible from sample to sample: in a duplicate run, peak *III* represented 76.5% and 69.9% of the total lipid phosphorus, and in a quadruplicate run, peak *IV* was 8.14%, 8.40%, 7.35%, and 5.40%, while peak *V* was 19.6%, 20.6%, 17.5%, and 19.0% of the total phospholipid phosphorus. There appeared to be little overlap of fractions as evidenced by the characterizations of the five peaks as given below.

Peak I (*Noncholine*). This material containing 5.5 ± 0.2 S.E. per cent[6] of the total lipid phosphorus was eluted with 20% methanol. It was shown to be ninhydrin positive and Reinecke salt negative on paper strip. Its R_f on paper strip is the same as that recorded for phosphatidyle thanolamine and phosphatidyl serine (19).

Peak II. A fraction containing 1.4 ± 0.1 S.E. per cent of the total lipid phosphorus was eluted with 40% methanol. It had insufficient amounts of material to be fully characterized, but preliminary analyses showed it to be all noncholine-containing material.

Peak III (*Lecithin*). The peak *III* material, containing 66.6 ± 1.2 S.E. per cent of the total lipid phosphorus, also was eluted with 40% methanol. In two phospholipid separations the amounts of phosphorus of peak *III* in the aqueous phase obtained after saponification and extraction with chloroform (24) were 98.3% and 97.2% of the total. In a series of five experiments the material in the peak was found to have a mean ester to phosphorus molar ratio of 1.89 ± 0.02 S.E. In one experiment the choline to phosphorus molar ratio was 1.02, and the glycerol to phosphorus was 0.98. As further evidence of identity and purity, the entire lecithin fraction, when subjected to rechromatography, gave only one peak. Moreover, glass paper chromatography gave only one spot with an R_f of 0.77 which corresponded with an R_f of 0.75 for dog bile lecithin (Fig. 2).

Peak IV (*Sphingomyelin*). The peak *IV* material, representing 8.5 ± 0.5 S.E. per cent of the total lipid phosphorus, was eluted with 60% methanol. In two experiments the nonsaponifiable fraction of the eluted material was found to contain 88.3% and 92.6% of the total phosphorus. Furthermore, the R_f on glass paper

FIG. 2. Silicic acid-impregnated glass paper chromatography of *1*, peak *III*; *2*, peak *IV*; and *3*, peak *V* compared to the standards *4*, lecithin; *5*, sphingomyelin; *6*, lysolecithin; and *7*, a mixture of the three standards. The spots represent 20 μg of phospholipid from the complete pooled peaks derived from four column fractionations. This amount of phospholipid represents an overload in order to detect small quantities of impurities. As little as 0.1 μg phospholipid is detectable by the charring technique. The top of the photograph represents the solvent front. The solvent mixture was phenol-diethyl ether-acetone-water.

[6] All percentages ± S.E. of total phospholipid P are based on a sample of 15 rat plasmas.

was 0.62, and the R_f of sphingomyelin[7] was 0.63. Another spot with the R_f of lecithin was barely discernible (Fig. 2).

Peak V. (*Lysolecithin*). This peak was eluted with pure methanol. This fraction was the second largest and contained 17.5 ± 0.6 S.E. per cent of the total lipid phosphorus. In two experiments the phospholipid was found to be 96.3% and 95.2% saponifiable. In a series of three experiments the ester to phosphorus ratios were 0.95, 1.03, and 0.88. In two experiments the molar ratio of choline to phosphorus was determined and was found to be 0.98 and 1.00, whereas glycerol to phosphorus was 1.11 and 0.99. Paper chromatography showed only one spot with an R_f of 0.49, which corresponded to an R_f of 0.54 for lysolecithin prepared from dog bile lecithin (Fig. 2).

Experiments with Labeled Phospholipids. To test the possibility that lysolecithin eluted with 100% methanol is a breakdown product of lecithin, P^{32}-labeled phospholipids were prepared by injecting two rats intraperitoneally with 200 μc of P^{32}-phosphate each, and obtaining blood 18 hours later. Plasma lipids were extracted and phospholipids separated as described previously. The entire 40% methanol fraction, which in these instances showed only one peak, was evaporated *in vacuo*, extracted in chloroform, and rechromatographed as before. The entire P^{32} load appeared in the 40% methanol fraction with no discernible radioactivity in the 100% methanol eluate. The same type of experiment was performed with a sample of dog bile phospholipids, which is known to contain primarily lecithin. The first chromatographic separation of dog bile phospholipids revealed less than 2% of the total lipid phosphorus as lysolecithin phosphorus. Rechromatography of the lecithin fraction gave rise to less than 1% of the total phosphorus in the 100% methanol eluate. These experiments indicate that a lecithin fraction prepared without prior chromatography (dog bile) yields practically no lysolecithin when subjected to silicic-acid separations. In addition, when chromatographed dog bile or rat plasma lecithin fractions were taken to dryness, re-extracted, and again chromatographed, no breakdown of lecithin was observed. Apparently the procedures involved in the separation of phospholipids as described here are not sufficiently harsh to produce lysolecithin from lecithin as an artifact.

Another way of establishing the independent origin of plasma lysolecithin would be the determination of the specific activities of the lecithin and lysolecithin after P^{32} administration. Table 1 shows these results.

[7] Kindly supplied by Dr. H. E. Carter.

One animal killed 6 hours after administration of 100 μc of P^{32}-phosphate showed a lysolecithin specific activity only slightly lower than that of lecithin. Since, according to these data, lysolecithin could have been derived from *in vitro* hydrolysis of lecithin, the time interval after P^{32} administration was prolonged until at 18 to 20 hours all animals showed a considerable difference between the lecithin and lysolecithin specific activities. For all animals the difference was significant at $p < 0.001$. If lysolecithin were a simple *in vitro* breakdown product of lecithin, the specific activities of the two compounds should have been the same unless there were a selective breakdown of part of the lecithin with a specific activity lower than that represented by the whole lecithin fraction.

TABLE 1. SPECIFIC ACTIVITIES OF INDIVIDUAL PHOSPHOLIPIDS OF RAT PLASMA AT DIFFERENT TIME INTERVALS AFTER INJECTION OF P^{32}

Time Interval	Specific Activity (cpm/μg P)				Difference*
	Non Choline-Containing	Lecithin	Sphingomyelin	Lysolecithin	
hours					*per cent*
6	37.0	67.0	34.4	62.4	6.9
16	42.1	77.5	60.3	73.5	5.2
18	48.7	70.0	56.5	52.7	24.7
18	51.9	94.0	68.0	84.9	9.7
18	37.4	76.3	48.2	58.8	23.0
18	19.0	37.5	21.0	19.0	49.4
20	21.0	37.2	28.7	31.2	16.1

* $\dfrac{(\text{Specific activity of lecithin} - \text{specific activity of lysolecithin}) \times 100}{\text{specific activity of lecithin}}$

That inhomogeneity in P^{32}-labeled lecithin does occur has been shown by Collins (26), who found that upon countercurrent separation, the lecithin of rat liver 90 minutes after injection of P^{32} could be split into three fractions with differing specific activities. Similarly, Harris *et al.* (27) found that P^{32}-labeled rat liver lecithins in successive fractions from silicic-acid columns differed considerably in their specific activities and their fatty acid composition. To examine whether this heterogeneity is present in plasma, one rat was injected with P^{32}-phosphate, and 18 hours later blood was obtained for extraction and silicic-acid chromatography in the usual manner. Each 5-ml eluate was analyzed for P^{32} and P^{31}. The specific activities of the

lecithin fractions are shown in Figure 3. Apparently, as was observed in liver (26, 27), marked differences in the plasma lecithin specific activities exist. In the same plasma the lysolecithin fraction also showed differing specific activities which overlapped in part with those of lecithin. These data therefore cannot exclude the possibility that the lysolecithin was derived from the selective breakdown of a low specific activity lecithin.

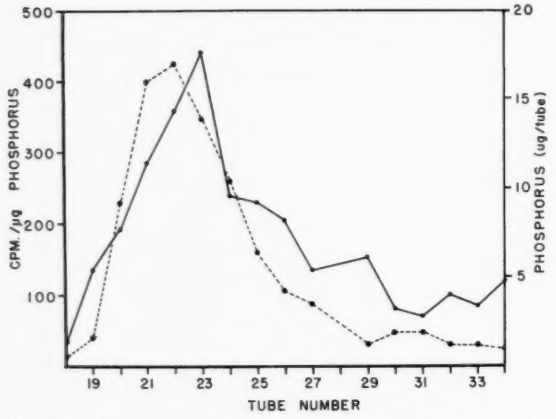


FIG. 3. Specific activities of rat plasma lecithin fractions eluted from silicic acid with 40% methanol. Broken line = phosphorus curve; unbroken line = specific activity curve.

Even if selective breakdown of P^{32}-lecithin on the silicic-acid column is assumed, such breakdown must be extremely rapid, as shown by the following experiment: Rat plasma lipids were extracted with chloroform-methanol 2:1 (v/v) for 5 minutes and concentrated *in vacuo*. Extraction and concentration were performed at 4°. A 5-µl portion of the concentrated lipids was spotted on silicic acid-impregnated glass paper and dried at room temperature for 1 minute. The paper was developed with diethyl ether-phenol-acetone-water within 20 minutes (19). Figure 4 shows a clear lysolecithin spot. Thus, if silicic acid were responsible for the breakdown of lecithin, this would have had to happen very early in the separation, since otherwise streaking of the spot would occur during chromatography.

Another way to test whether lecithin is converted to lysolecithin on the silicic-acid column would be to obtain a plasma phospholipid sample from which the free fatty acids had been removed. Such a sample, when subjected to column chromatography, should furnish free fatty acids only if lecithin breakdown occurred. Therefore 200 µc of acetate-1-C^{14} and 100 µc of P^{32}-phosphate[8] were injected intraperitoneally into a rat 6 hours prior to sacrifice. Plasma lipids were extracted at 4° and the neutral lipids dialyzed through a rubber membrane (28) as shown in Figure 5. The nondialyzable (phospholipid) fraction was found to be free of fatty acids by the glass paper chromatographic technique (29) utilizing 0.5% methanol in petroleum ether (b.p. 60°–70°, Skellysolve B). After chromatography of these phospholipids through the activated silicic-acid column, the chloroform eluate contained 56 cpm (Fig. 5). This could represent a slight contamination of the nondialyzable fraction by neutral lipids or a minimal liberation of fatty acids from phospholipids upon initial

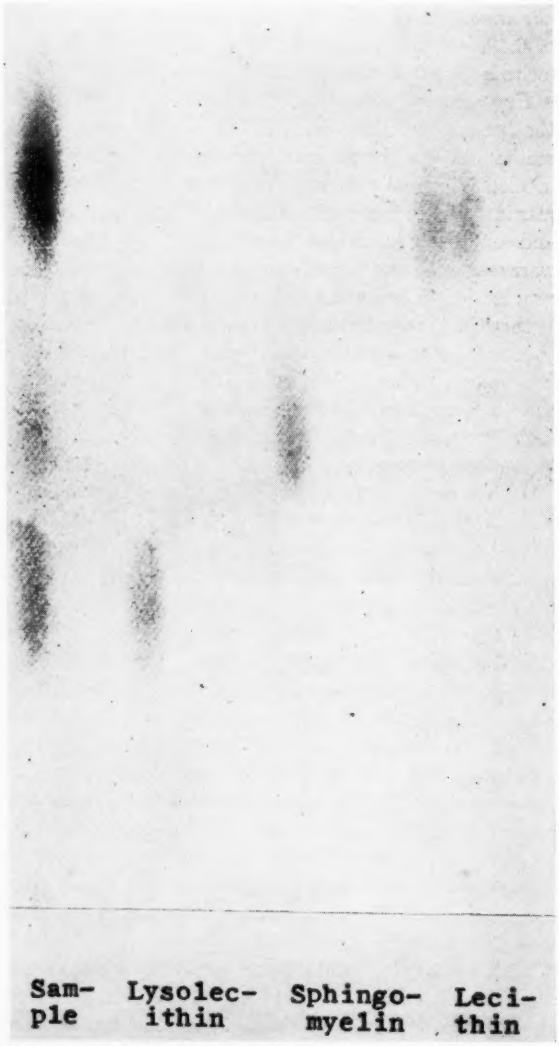
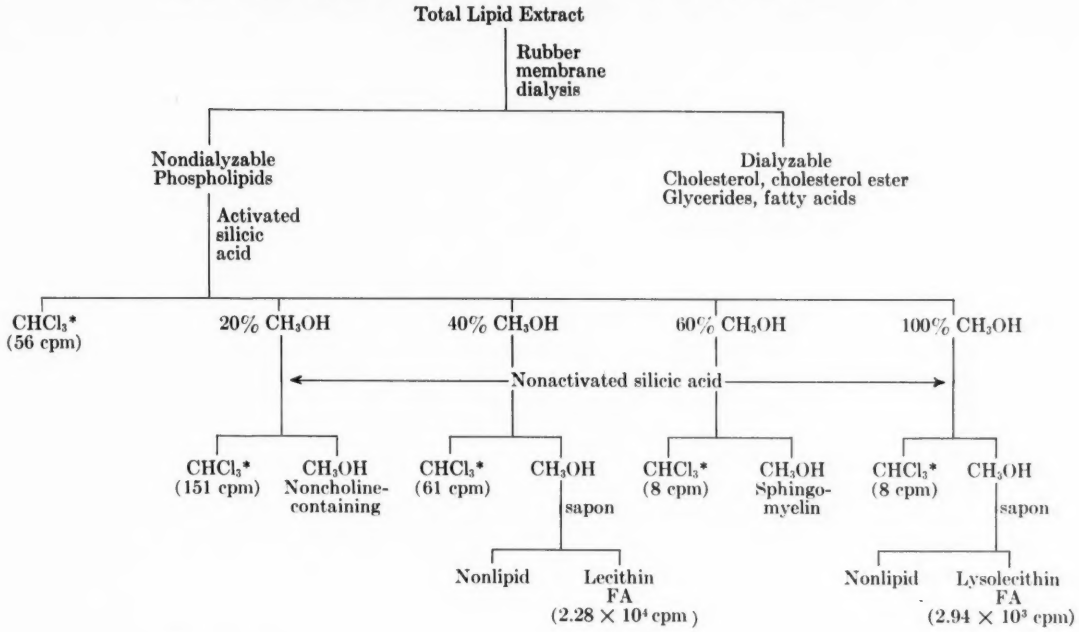


FIG. 4. Silicic acid-impregnated glass paper chromatography of a phospholipid sample briefly extracted from rat plasma with chloroform-methanol and standards of lysolecithin, sphingomyelin, and lecithin.

[8] The P^{32} merely served to monitor the eluates; see footnote 3.

FIG. 5. FLOWSHEET FOR COMBINED DIALYSIS-CHROMATOGRAPHY EXPERIMENT.



* Total C^{14} in chloroform eluates: 284 cpm.

contact with silicic acid. The 20%, 40%, 60%, and 100% methanol eluates were dried separately *in vacuo*, dissolved in chloroform, and rechromatographed with chloroform on nonactivated silicic acid-Super-Cel. The chloroform eluates, which would contain the fatty acids if any had been previously liberated on activated silicic acid, gave a total radioactivity of 228 cpm. The total C^{14} in the lecithin fatty acids liberated by hydrolysis (13) was 2.28×10^4 cpm. In the lysolecithin fatty acids 2.94×10^3 cpm were found. However, in the combined chloroform eluates only 284 cpm appeared, which could represent breakdown of lecithin to lysolecithin. If the specific activities of the α and β fatty acids of lecithin are assumed to be of the same order of magnitude, the C^{14} in the chloroform eluates would at most represent only a small amount of free fatty acids and hence account for only a small portion of the lysolecithin found in rat plasma.

DISCUSSION

Our characterization of the phospholipid fractions separated from rat plasma is indicative of the high purity of lecithin and lysolecithin, although the sphingomyelin was only 90% pure. The Phillips column (2, 3) was tried in our laboratory, but it separated lecithin and sphingomyelin poorly: the lecithin fraction contained considerable quantities of nonsaponifiable phospholipid, presumably sphingomyelin, and the sphingomyelin was also contaminated with lecithin. Furthermore, Phillips found that his lysolecithin was only 79% pure, the contaminants being about 10% sphingomyelin and lecithin each.

The proof of purity of lecithin and lysolecithin obtained from our column depends not only on the chemical characterizations but is also evidenced by rechromatography of these fractions on silicic acid-impregnated glass paper by a solvent system that differed from the one used in the column chromatography. Even overloading the paper by a factor of ten (Fig. 2) failed to show impurities in these two fractions.

The finding of plasma lysolecithin in rat plasma parallels the results of Phillips (2, 3) and Gjone *et al.* (1) in human plasma. However, Hanahan *et al.* (8) reported analyses of human plasma phospholipids in which no lysolecithin was found. This investigator suggested that his failure to find lysolecithin might have resulted from differences in extraction techniques or the use of a different anticoagulant. For this reason a human blood sample was treated with ACD[9]

[9] ACD: Abbott Laboratories.

and extracted with either chloroform-methanol 2:1 (v/v) or ethanol-diethyl ether 3:1 (v/v), the solvent system used by Hanahan. The fractionation on our activated silicic acid gave lysolecithin contents of 10.8% of the lipid phosphorus in the chloroform-methanol extract, and 8.4% of lipid phosphorus in the ethanol-diethyl ether. The lysolecithin content of a rat plasma sample extracted by ethanol-diethyl ether was also about the same as that observed previously in other samples after chloroform-methanol extraction. Another factor which could affect the hydrolysis of a lysolecithin precursor is temperature, but separation of rat plasma by centrifugation for 10 minutes at 4° or room temperature, and subsequent extraction of rat plasma lipids at either of these temperatures, gave the same lysolecithin content. Therefore it seems highly unlikely that lysolecithin could have been formed as an artifact in the interval between removal of blood from the rat and the extraction of lipids from plasma.

The presence of lysolecithin in various biological materials has been asserted by different authors. However, in view of the known hemolytic activity of lysolecithin, its presence in blood in appreciable quantities has been doubted. Neither Hanahan *et al.* (8) nor Nelson and Freeman (6) were able to discover lysolecithin in human blood serum. Douste-Blazy (30), however, found an appreciable quantity of lysolecithin in one sample of human plasma phospholipids studied by countercurrent distribution. Similarly, Hajdu *et al.* (31) reported the existence of lysolecithin in human plasma. Phillips (2, 3) and Gjone *et al.* (1) demonstrated the presence of lysolecithin in human serum by silicic-acid column chromatography, and Marinetti *et al.* (4, 5) confirmed this finding by the application of paper chromatography. Ayrault-Jarrier (32) found lysolecithin in the low density lipoprotein of human serum. Phillips (33), moreover, compared various serum lipoproteins and found relatively more lysolecithin in the high density lipoprotein fraction ($d > 1.063$) than in the low density fraction ($d < 1.063$), while the highest lysolecithin relative to other phospholipids occurred in the protein fraction of density higher than 1.21 (34).

One might ask whether possibly the decomposition of plasmalogens, which are known to be prone to breakdown, could account for the presence of lysolecithin. To study this possibility, a rat serum sample was analyzed immediately after extraction for plasmalogen by the method of Leupold and Büttner (35). Less then 3% of the plasma lipid phosphorus could be found as plasmalogen, and therefore cannot account to even a small extent for the presence of lysolecithin.

The clear separation of lysolecithin from briefly extracted rat plasma by paper chromatography speaks against the concept that lysolecithin is an *in vitro* breakdown product of lecithin. Furthermore, the absence of a lysolecithin peak when lecithin was rechromatographed, and the insignificant liberation of C^{14}-fatty acids from rat plasma phospholipids during silicic-acid chromatography, do not support the hypothesis that lecithin breaks down to lysolecithin during column chromatography. Finally, the difference in specific activities of serum lysolecithin and lecithin would be evidence against a random breakdown of lecithin to lysolecithin *in vitro*. It thus appears almost certain that lysolecithin is a normal constituent of rat plasma.

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REFERENCES

1. Gjone, E., J. F. Berry and D. A. Turner. *J. Lipid Research* **1**: 66, 1959.
2. Phillips, G. B. *Proc. Natl. Acad. Sci. U.S.* **43**: 566, 1957.
3. Phillips, G. B. *Biochim. et Biophys. Acta* **29**: 594, 1958.
4. Marinetti, G. V., M. Albrecht, T. Ford, and E. Stotz. *Biochim. et Biophys. Acta* **36**: 4, 1959.
5. Marinetti, G. V., and E. Stotz. *Biochim. et Biophys. Acta* **37**: 571, 1960.
6. Nelson, G. J., and N. K. Freeman. *J. Biol. Chem.* **234**: 1375, 1959.
7. Hirsch, J., and E. H. Ahrens, Jr. *J. Biol. Chem.* **233**: 311, 1958.
8. Hanahan, D. J., R. M. Watts and D. Pappajohn. *J. Lipid Research* **1**: 421, 1960.
9. Hanahan, D. J., J. C. Dittmer and E. Warashina. *J. Biol. Chem.* **228**: 685, 1957.
10. Folch, J., M. Lees and G. H. Sloane Stanley. *J. Biol. Chem.* **226**: 497, 1957.
11. Bartlett, G. R. *J. Biol. Chem.* **234**: 466, 1959.
12. McKay, B. P., and D. B. Zilversmit. *Nucleonics* **11**: 58, 1953.
13. Dawson, R. M. C. *Biochem. J.* **57**: 237, 1954.
14. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50**: 152, 1957.
15. Carlson, L. A., and L. B. Wadström. *Clin. Chim. Acta* **4**: 197, 1959.
16. Frisell, W. R., and C. G. Mackenzie. In *Methods of Biochemical Analysis*, edited by David Glick, New York, Interscience Publishers, Inc., 1958, vol. 6, p. 70.
17. Burmaster, C. F. *J. Biol. Chem.* **164**: 233, 1946.
18. Long, C., and I. F. Penny. *Biochem. J.* **65**: 382, 1957.
19. Brown, M., D. A. Yeadon, L. A. Goldblatt, and J. W. Dieckert. *Anal. Chem.* **29**: 30, 1957.
20. Dieckert, J. W., W. B. Carney, R. L. Ory, and N. J. Morris. *Anal. Chem.* **30**: 1442, 1958.
21. Hack, M. H. *Biochem. J.* **54**: 602, 1953.
22. Rapport, M. M., and N. Alonzo. *J. Biol. Chem.* **217**: 193, 1955.

23. Wheeldon, L. W., and F. D. Collins. *Biochem. J.* **70:** 43, 1958.
24. McCandless, E., and D. B. Zilversmit. *Arch. Biochem. Biophys.* **62:** 402, 1956.
25. Berenblum, I., and E. Chain. *Biochem. J.* **32:** 295, 1938.
26. Collins, F. D. *Nature* **186:** 366, 1960.
27. Harris, P. M., D. S. Robinson and G. Getz. *Nature* **188:** 742, 1960.
28. Van Beers, G. J., H. de Iongh and J. Boldingh. In *Essential Fatty Acids*, edited by H. M. Sinclair, New York, Academic Press, Inc.; London, Butterworths Scientific Publications, 1958, p. 43.
29. Newman, H. A. I., E. L. McCandless and D. B. Zilversmit. *J. Biol. Chem.* **236:** 1264, 1961.
30. Douste-Blazy, L. *Compt. rend.* **239:** 460, 1954.
31. Hajdu, S., H. Weiss and E. Titus. *J. Pharmacol. Exptl. Therap.* **120:** 99, 1957.
32. Ayrault-Jarrier, M. Thesis, University of Paris, 1959.
33. Phillips, G. B. *J. Clin. Invest.* **38:** 489, 1959.
34. Phillips, G. B. *Proc. Soc. Exptl. Biol. Med.* **100:** 19, 1959.
35. Leupold, F., and H. Büttner. *Z. physiol. Chem., Hoppe-Seyler's* **292:** 13, 1953.

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Dietary alterations of fatty acids of erythrocytes and mitochondria of brain and liver*

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SUMMARY

The fatty acid composition of erythrocyte and liver mitochondrial lipids was readily and drastically altered by varying the fatty acid content of the diet. Nonessential polyunsaturated fatty acids were found in these tissues when the tissue level of linoleic acid fell below 10% of the total fatty acids. In essential fatty acid deficiency, two isomeric eicosatrienoic acids appeared, except when the diet supplied other more highly unsaturated nonessential fatty acids. Although brain mitochondrial lipids were relatively less affected by dietary manipulations, their fatty acid compositions could be significantly altered by variations of dietary fat; nonessential polyunsaturated fatty acids from cod liver oil were also incorporated into these lipids. It is postulated that such alterations in the polyunsaturated fatty acid content of phospholipids, other lipid complexes, or both, may be of importance in metabolism.

Essential fatty acid deficiency has been described in the rat, mouse, dog, child, and a moth larva (1). Recently it has been pointed out that a high intake of polyunsaturated fatty acids, inadequately protected against autoxidation *in vivo*, facilitates the appearance of encephalomalacia in the chick (2 to 5) and in man (6), nutritional dystrophy in the rat (7), and peroxide hemolysis of human erythrocytes (8, 9, 10). The present report attempts to define in biochemical terms the marginal state of essential fatty acid deficiency and the criteria for determination of this state in individual tissues.

Although it has been shown that the turnover of unsaturated fatty acids in rat liver and carcass is high (11), it has been assumed that the fatty acids of the central nervous system, particularly the brain, turn over far more slowly. However, a previous report (10) has shown that the fatty acid composition of human erythrocytes and chick cerebella were readily altered by variations in dietary fats. In the present investigation the lipids from various tissues of the rat were surveyed to evaluate alterations in fatty acid composition produced by qualitative and quantitative changes in dietary lipids. Tissues containing most of their lipid as triglyceride were not studied, since it has been long recognized (12, 13) that depot fats readily take on the characteristics of the ingested fat. Rather, formed elements of definite structural character, such as brain and liver mitochondria and erythrocyte stroma, were chosen for study. The lipids of these tissues contain most of their fatty acids in the form of phospholipids (see Results), and it was considered adequate for the purposes of this study to analyze these fatty acids without further subfractionation into phospholipid classes. In these studies attention was focused on three effects produced by dietary manipulations: changes in essential fatty acid levels, appearance of nonessential polyunsaturated fatty acids as a criterion of essential fatty acid deficiency, and incorporation of dietary acids (14, 15).

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EXPERIMENTAL

Preparation of Animals and Tissue Lipids. Weanling rats of the Sprague-Dawley strain were raised on synthetic diets (Table 1) containing one of the following fats substituted isocalorically for carbohydrate: 15% coconut oil, 15% corn oil, 7% cod liver oil, and 0.2% corn oil. The latter diet was intended to be a "fat-

TABLE 1. EXPERIMENTAL DIETS

Ingredient	15% Fat by Weight		7% Fat by Weight		0.2% Fat by Weight	
	% of calories	*% by weight*	*% of calories*	*% by weight*	*% of calories*	*% by weight*
Casein (vitamin-test)	21.8	24.8	21.8	22.8	21.8	20.9
Dextrose	26.2	29.8	34.0	35.5	41.5	40.0
Cornstarch	22.7	25.8	28.9	30.4	36.5	35.1
Coconut oil or corn oil*	29.4	15.0				
Cod liver oil*			15.3	7.0		
Low fat (as corn oil)					0.2	0.2
	g/1,000 cal.	*% by weight*	*g/1,000 cal.*	*% by weight*	*g/1,000 cal.*	*% by weight*
Choline dihydrogen citrate	0.72	0.33	0.72	0.30	0.72	0.27
Vitamin mix†	0.72	0.33	0.72	0.30	0.72	0.27
Salts (USP XIV)	8.7	4.0	8.7	3.7	8.7	3.3

* Major components of dietary oils: Coconut oil 12:0—28%; 14:0—28%; 16:0—17%; 18:0—6%; 18:1—14%; 18:2—4%. Cod liver oil 16:0—8%; 16:1—9%; 18:1—18%; 20:1—13%; 20:4—7%; 20:5—15%; 22:6—12%. Corn oil 16:0—8%; 18:1—30%; 18:2—54%.

† Vitamin mix contains i-inositol, 24.1 mg, para-aminobenzoic acid, 24.1 mg, calcium pantothenate, 13.5 mg, 2-methyl-1, 4-naphthoquinone, 10.9 mg, niacin, 21.8 mg, thiamine HCl, 4.8 mg, pyridoxine-HCl, 4.8 mg, riboflavin, 4.8 mg, folic acid, 0.4 mg, biotin, 98 μg, vitamin B_{12}, 6.5 μg, and starch to 0.72 g. Vitamins A (2,500 I.U.) and D (360 I.U.) were given weekly as Oleum Percomorphum drops.

free" diet with sufficient lipid to meet requirements for essential fatty acids. Animals were sacrificed at zero time, 6 weeks, and 21 weeks.

Erythrocytes were lysed and extracted by the procedure of Morris *et al.* (16). Liver and brain were homogenized, and mitochondria isolated by conventional techniques, employing differential centrifugation between 550 and 11,000 $\times$ *g*, as described in the second procedure of Brody and Bain (17). Lipid extracts were obtained as noted above (16). DL-α-tocopherol was added to the extracting solvents and was found to follow the lipid throughout subsequent procedures. All evaporations were performed under nitrogen, and samples were stored in ten times their weight of redistilled petroleum ether under a nitrogen atmosphere at 0°. Methyl esters were obtained by a modification of the method of Stoffel *et al.* (18). Fatty acid esters were determined by the method of Clayton *et al.* (19), and nitrogen by the method of Hiller (20).

Gas-Liquid Chromatography. Fatty acid compositions were determined by gas-liquid chromatography of methyl esters on Celite® columns utilizing Apiezon M, ethylene glycol adipate polyester, ethylene glycol succinate polyester, and silicone grease as liquid phases (Table 2). Most of the reported data were obtained on ethylene glycol succinate polyester columns. Standard mixtures were used to calibrate area measurements. Since ethylene glycol succinate polyester columns do not resolve linolenic acid (18:3) from eicosaenoic acid (20:1) at 170° (21), representative samples were analyzed on ethylene glycol adipate polyester columns. The latter columns clearly resolve these components, but linolenic acid (18:3) was not detected in any of the tissues examined. Structural assignments are based largely on the data of Farquhar *et al.* (22).

The $\Delta^{5,8,11,14,17}$ eicosapentaenoic acid (20:5) and $\Delta^{7,10,13,16,19}$ docosapentaenoic acid (22:5) from cod liver oil (23, 24) fit the data of Farquhar *et al.* (22) better than do the corresponding isomers of these acids found normally in rat tissue lipids. Mead and Slaton (25) have found the major trienoic acid present in an essential fatty acid deficiency to be the $\Delta^{5,8,11}$ eicosatrienoic acid (20:3), while the $\Delta^{7,10,13}$ eicosatrienoic acid (20:3) (26) was found only as a minor constituent. In this paper two peaks on the gas-liquid chromatogram are designated as eicosatrienoic acids (20:3) and their total

TABLE 2. CONDITIONS FOR GAS-LIQUID CHROMATOGRAPHIC ANALYSIS

Liquid Phase	Apiezon M	Ethylene Glycol Adipate Polyester	Ethylene Glycol Succinate Polyester*	Silicone Grease
Percentage of liquid phase by weight	20%	20%	17%	20%
Support	Celite	Celite	Chromosorb W	Celite
Mesh	150–200	140–200	80–100	60–80
Column	glass	glass	glass	stainless steel
Internal diameter (mm)	1	4	6	6
Length (cm)	240	120	240	60
Temperature (°)	190	165	170	240
Carrier gas	argon	argon	argon	helium
Pressure (psi)	90	20	65	20
Flow rate (cc/min)	16	37.5	200	60
Detector	radium-226 strontium-90	radium-226 strontium-90	tritium	thermal conductivity
Detector current (amps)	3×10^{-8} at 1200 v	3×10^{-8} at 1200 v	3×10^{-8} at 900 v	2×10^{-1} at 12 v

* Applied Science Laboratories, Inc., State College, Pa.

is in reasonable agreement with the triene content of the samples, as determined by alkali isomerization using the extinction coefficients for eicosatrienoic acid (20:3), determined by Holman and Hayes (27). Oxidation experiments are now in progress to determine which peak contains the $\Delta^{5,8,11}$ eicosatrienoic acid (20:3). Assignments of 16:al and 18:al designate saturated aliphatic aldehydes. Data supporting these characterizations will be presented in detail in a future paper. Values for arachidonic acid (20:4) are slightly high, since small amounts of behenic acid (22:0) contaminated the 20:4 peak on ethylene glycol succinate polyester columns.

RESULTS

Fatty Acids of Liver Mitochondrial Lipids. The fatty acids of liver mitochondrial lipids were analyzed as methyl esters by gas-liquid chromatography (Table 3). Five rats were sacrificed for each set of conditions. In weanlings the mitochondrial lipids contained approximately 14% linoleic acid (18:2), 16% arachidonic acid (20:4), and 5% docosahexaenoic acid (22:6). By varying the nature of the dietary lipid, the linoleic acid (18:2) content of liver mitochondrial lipids could be lowered to 5% or raised to 20%, while arachidonic acid (20:4) could be varied from 5% to 24%, and docosahexaenoic acid (22:6) from 2% to 14%. Rat liver mitochondrial lipids contained 93% phospholipid (28), and the fatty acids of this phospholipid might be expected to reflect the composition of the dietary fatty acids.

Direct incorporation of dietary fatty acids was evident in the cod liver oil group in which 9% to 15% of total liver mitochondrial lipids consisted of pentaenoic acids ($\Delta^{5,8,11,14,17}$ 20:5 and $\Delta^{7,10,13,16,19}$ 22:5). An increase in concentration of the eicosatrienoic acids (20:3) was noted in the groups receiving 0.2% corn oil or 15% coconut oil.

Fatty Acids of Erythrocyte Lipids. The stromal lipids of erythrocytes from weanling rats were found to contain 6% linoleic acid (18:2), 11% arachidonic acid (20:4), and 2% docosahexaenoic acid (22:6) (Table 4). Mead and Fillerup (29) found 1% neutral lipid, 30% cholesterol, and 70% phospholipid plus cerebroside in the lipids of rat erythrocytes. Alteration of dietary lipids lowered the linoleic acid (18:2) of erythrocyte lipids to 4%, or raised it to 13%. Similarly, arachidonic acid (20:4) was found to vary from 7% to 23%, and docosahexaenoic acid (22:6) from 1.5% to 6%. Sixteen per cent of the total erythrocyte fatty acids consisted of pentaenoic acids ($\Delta^{5,8,11,14,17}$ 20:5 and $\Delta^{7,10,13,16,19}$ 22:5) in the group receiving cod liver oil. Eicosatrienoic acids (20:3) increased in concentration in the groups receiving 0.2% corn or 15% coconut oil.

Fatty Acids of Brain Mitochondrial Lipids. In

TABLE 3. FATTY ACID COMPOSITION OF LIVER MITOCHONDRIAL LIPIDS

Fatty Acid	Weanlings at Start of Experiment	Animals on Diets 6 Weeks				Animals on Diets 21 Weeks			
		0.2% Corn Oil	15% Coconut Oil	15% Corn Oil	7% Cod Liver Oil	0.2% Corn Oil	15% Coconut Oil	15% Corn Oil	7% Cod Liver Oil
12:0	0.5* ± 0.3†	0.9 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.6 ± 0.3	0.1 ± 0.0	0.4 ± 0.2	0.3 ± 0.2	trace‡
14:0	1.1 ± 0.2	2.6 ± 0.6	2.6 ± 0.6	2.0 ± 0.4	2.1 ± 1.0	0.8 ± 0.2	2.1 ± 0.6	1.2 ± 0.6	0.9 ± 0.2
16:0	18.5 ± 1.2	18.5 ± 2.0	19.8 ± 1.8	17.3 ± 2.6	24.5 ± 3.4	22.1 ± 3.4	17.5 ± 3.0	12.2 ± 2.4	21.6 ± 7.4
16:1	1.5 ± 0.6	7.1 ± 1.0	4.5 ± 0.6	1.7 ± 0.2	5.5 ± 0.6	5.5 ± 1.4	6.5 ± 1.0	2.0 ± 0.6	6.5 ± 1.6
18:0	21.7 ± 1.4	18.3 ± 0.6	19.7 ± 1.2	20.1 ± 3.4	17.5 ± 1.6	16.1 ± 1.2	14.7 ± 2.0	12.1 ± 3.4	12.4 ± 1.2
18:1	14.7 ± 1.4	22.7 ± 1.4	19.5 ± 2.4	12.9 ± 0.8	17.6 ± 1.0	20.3 ± 2.2	18.5 ± 3.0	15.2 ± 2.8	14.3 ± 1.2
18:2	14.1 ± 0.8	6.3 ± 0.4	9.5 ± 1.0	20.3 ± 2.4	5.8 ± 1.2	7.7 ± 1.4	10.1 ± 3.0	20.9 ± 3.6	5.0 ± 1.4
20:0	1.0 ± 0.8	0.4 ± 0.2	0.4 ± 0.4	0.5 ± 0.2	0.4 ± 0.4	0.1 ± 0.0	0.3 ± 0.2	0.3 ± 0.3	0.1 ± 0.1
20:1	0.4 ± 0.3	0.9 ± 0.2	0.8 ± 0.2	0.9 ± 0.2	1.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.2	0.4 ± 0.1	1.6 ± 1.0
20:?		0.5 ± 0.0		1.5 ± 0.4		0.1 ± 0.1		0.7 ± 0.3	0.3 ± 0.0
20:3		5.7 ± 1.0	3.9 ± 0.2	trace		3.1 ± 1.4	2.3 ± 0.8		
20:3		1.3 ± 0.2	1.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	1.4 ± 0.6	2.1 ± 0.6	0.6 ± 0.3	0.5 ± 0.2
20:4	16.0 ± 1.6	9.2 ± 1.0	11.4 ± 1.6	17.5 ± 1.4	5.4 ± 0.8	14.8 ± 3.6	17.1 ± 3.0	25.1 ± 4.4	5.7 ± 1.0
20:5	0.7 ± 0.5	0.7 ± 0.2		0.3 ± 0.3		0.6 ± 0.3	1.1 ± 1.0	0.7 ± 0.1	0.6 ± 0.4
20:5§					6.9 ± 0.8	1.0 ± 0.3			12.0 ± 1.6
22:2						0.4 ± 0.3	0.3 ± 0.2	0.5 ± 0.4	0.3 ± 0.3
22:5	4.3 ± 1.6	1.0 ± 0.4	1.1 ± 0.2	0.9 ± 0.6		1.3 ± 0.4	1.3 ± 0.6	1.7 ± 0.8	0.3 ± 0.3
22:5‖					2.2 ± 0.4	0.5 ± 0.2		0.4 ± 0.0	3.5 ± 1.2
22:6	5.2 ± 1.0	1.8 ± 0.2	3.0 ± 1.0	1.6 ± 0.6	7.9 ± 0.4	5.3 ± 1.4	4.1 ± 1.4	3.5 ± 1.6	14.0 ± 3.2
24:0		0.5 ± 0.3	0.8 ± 0.4	1.3 ± 0.6	1.2 ± 0.6	0.5 ± 0.5	0.3 ± 0.2	0.8 ± 0.3	
mg ester/mg nitrogen in mitochondria		0.8 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.1

* Percentage of total area of a gas-liquid chromatographic elution diagram.
† Standard deviation (5 samples).
‡ Present, but less than 0.05%.
§ $\Delta^{5,8,11,14,17}$ 20:5 from cod liver oil.
‖ $\Delta^{7,10,13,16,19}$ 22:5 from cod liver oil.

TABLE 4. FATTY ACID COMPOSITION OF ERYTHROCYTE LIPIDS. ANIMALS ON DIETS 21 WEEKS

Fatty Acid	Weanlings at Start of Experiment	0.2% Corn Oil	15% Coconut Oil	15% Corn Oil	7% Cod Liver Oil
12:0	0.3* ± 0.2†		0.3 ± 0.2		
14:0	2.3 ± 1.3	0.9 ± 0.4	1.7 ± 0.2	0.9 ± 0.3	0.7 ± 0.2
16:al‡	2.2 ± 0.6	3.0 ± 1.0	3.0 ± 0.4	2.6 ± 0.2	3.3 ± 0.2
16:0	16.7 ± 2.7	20.9 ± 4.4	15.1 ± 4.4	21.6 ± 3.8	22.9 ± 4.6
16:1	6.4 ± 2.3	2.1 ± 0.6	1.3 ± 0.4	0.6 ± 0.3	2.4 ± 0.6
18:al‡	1.0 ± 0.5	2.4 ± 0.6	2.6 ± 0.8	2.2 ± 0.2	2.8 ± 0.2
18:0	11.9 ± 1.7	16.4 ± 2.0	15.5 ± 2.2	16.5 ± 1.2	13.9 ± 1.0
18:1	12.1 ± 0.5	14.8 ± 1.2	13.7 ± 1.4	11.4 ± 1.4	15.7 ± 1.0
18:2	5.7 ± 1.3	5.3 ± 1.0	8.1 ± 0.6	13.3 ± 1.4	3.9 ± 0.6
20:0	0.7 ± 0.3				
20:1	1.6 ± 1.0	0.6 ± 0.1	0.8 ± 0.5	0.4 ± 0.2	1.7 ± 0.6
20:?	0.7 ± 0.6			0.5 ± 0.2	
20:3	1.0 ± 0.4	2.4 ± 1.0	1.3 ± 0.4		
20:3		0.7 ± 0.2	1.2 ± 0.6	0.4 ± 0.3	0.4 ± 0.2
20:4	11.4 ± 2.1	22.9 ± 3.0	22.6 ± 2.0	23.3 ± 5.2	7.1 ± 0.8
20:5	1.4 ± 0.8	0.7 ± 0.5	1.3 ± 0.8	1.3 ± 0.6	
20:5§	2.1 ± 1.5				13.0 ± 2.4
22:2		0.5 ± 0.2	0.4 ± 0.2	0.8 ± 0.6	0.4 ± 0.2
22:5	2.4 ± 0.9	1.5 ± 1.2	1.9 ± 0.6	0.8 ± 0.2	1.1 ± 0.2
22:5‖	2.3 ± 0.9				3.6 ± 0.8
22:6	1.7 ± 0.2	1.5 ± 0.2	3.6 ± 2.4	0.8 ± 0.4	5.7 ± 1.2
24:0	3.1 ± 0.5	1.7 ± 0.8	2.8 ± 1.8	2.7 ± 0.4	0.5 ± 0.2

* Percentage of total area of gas-liquid chromatographic elution diagram.
† Standard deviation (5 samples; last column, 4 samples).
‡ Saturated aliphatic aldehydes.
§ $\Delta^{5,8,11,14,17}$ 20:5 from cod liver oil.
‖ $\Delta^{7,10,13,16,19}$ 22:5 from cod liver oil.

this laboratory the approximate composition of brain mitochondrial lipids has been found to be 22% cholesterol, 7% cholesterol ester, and 71% phospholipid. The lipids of brain mitochondria from weanling rats contained 1.7% linoleic acid (18:2), 11% arachidonic acid (20:4), and 9% docosahexaenoic acid (22:6) (Table 5). By varying the fatty acid composition of the dietary lipid, it was possible to lower the linoleic acid (18:2) content of the brain mitochondrial lipids to 0.5%. The arachidonic acid (20:4) levels could be varied from 8% to 13%, and docosahexaenoic acid (22:6) from 9% to 17%. Incorporation of pentaenoic acid isomers ($\Delta^{5,8,11,14,17}$ 20:5 and $\Delta^{7,10,13,16,19}$ 22:5) from cod liver oil into brain mitochondrial lipids was noted. Eicosatrienoic acids (20:3) did not appear to increase in concentration in the groups receiving 0.2% corn oil or 15% coconut oil.

DISCUSSION

From the experiments described above it is quite evident that the fatty acid composition of liver mitochondrial lipids can be altered by dietary fatty acids. Definite changes in the essential fatty acid content of mitochondrial lipids were observed, and direct incorporation of dietary fatty acids could be seen in the group receiving cod liver oil. On diets marginally deficient in essential fatty acids, eicosatrienoic acids (20:3) were found to increase in concentration.

Whereas it was originally thought that the stroma of the erythrocyte would represent a fatty acid composition intermediate in stability between liver mitochondria and brain mitochondria, this was not found to be the case in young animals. It was apparent that the lipids of the erythrocyte respond just as positively to alterations of dietary fatty acids as do liver mitochondria.

Brain mitochondrial lipids were more resistant to change than were the lipids of either of the other tissues studied, but even this fatty acid composition could be significantly altered by dietary fat. Direct incorporation of pentaenoic acids ($\Delta^{5,8,11,14,17}$ 20:5 and $\Delta^{7,10,13,16,19}$ 22:5) could be demonstrated when the diet contained cod liver oil. The essential fatty acid requirements of brain mitochondria were apparently met, as judged by their content of nonessential polyunsaturated fatty acids, even when other tissues in the

TABLE 5. FATTY ACID COMPOSITION OF BRAIN MITOCHONDRIAL LIPIDS

Fatty Acid	Weanlings at Start of Experiment	Animals on Diets 6 Weeks				Animals on Diets 21 Weeks			
		0.2% Corn Oil	15% Coconut Oil	15% Corn Oil	7% Cod Liver Oil	0.2% Corn Oil	15% Coconut Oil	15% Corn Oil	7% Cod Liver Oil
12:40	0.3* ± 0.1†	0.3 ± 0.2	0.3 ± 0.2	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
14:0	0.7 ± 0.1	0.6 ± 0.3	1.2 ± 0.6	0.6 ± 0.3	0.6 ± 0.3	0.8 ± 0.5	0.8 ± 0.4	0.5 ± 0.3	0.7 ± 0.4
16:al‡	3.2 ± 0.4	2.5 ± 0.2	2.0 ± 1.2	2.6 ± 0.6	2.7 ± 1.0	2.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.2	2.2 ± 0.6
16:0	19.6 ± 2.8	18.1 ± 1.8	17.1 ± 2.6	16.8 ± 1.2	16.6 ± 1.4	15.1 ± 2.2	14.8 ± 1.0	18.5 ± 1.2	14.3 ± 0.8
16:1	1.5 ± 0.6	1.6 ± 0.8	1.7 ± 0.6	0.8 ± 0.1	1.2 ± 0.2	0.8 ± 0.4	0.9 ± 0.4	0.5 ± 0.2	0.9 ± 0.6
18:al‡	2.8 ± 1.0	4.6 ± 2.6	2.7 ± 0.4	3.0 ± 1.0	3.3 ± 1.4	3.3 ± 0.8	3.7 ± 0.6	3.8 ± 0.8	3.4 ± 0.6
18:0	20.9 ± 0.8	19.4 ± 1.2	20.0 ± 1.8	17.6 ± 1.4	19.0 ± 0.8	20.7 ± 2.0	20.3 ± 2.2	21.2 ± 2.0	18.7 ± 2.0
18:1	20.8 ± 1.0	24.2 ± 3.0	23.3 ± 5.6	19.5 ± 3.2	21.9 ± 2.8	24.1 ± 2.6	21.7 ± 1.6	22.1 ± 2.0	22.8 ± 1.4
18:2	1.7 ± 0.2	0.6 ± 0.4	0.5 ± 0.2	1.3 ± 0.2	0.6 ± 0.2	0.5 ± 0.7	0.9 ± 0.2	1.3 ± 0.2	0.8 ± 0.4
20:0	1.3 ± 0.6	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.4 ± 0.1
20:1		1.7 ± 0.6	1.8 ± 0.6	1.6 ± 0.2	1.6 ± 0.6	2.9 ± 0.6	3.0 ± 0.6	3.0 ± 0.4	2.8 ± 0.4
20:?						0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.2	
20:3		0.5 ± 0.2	0.3 ± 0.3			0.5 ± 0.2	0.1 ± 0.1		0.1 ± 0.1
20:3	0.6 ± 0.5		0.5 ± 0.4	0.3 ± 0.2	0.4 ± 0.2	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.3
20:4	11.4 ± 1.2	9.5 ± 1.4	9.2 ± 3.2	12.8 ± 0.4	8.4 ± 0.8	9.3 ± 1.8	10.7 ± 0.8	10.2 ± 1.4	8.0 ± 1.0
20:5						0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	
20:5§									0.7 ± 0.2
22:2						0.4 ± 0.3	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.3
22:5	1.8 ± 1.0	1.6 ± 0.4	2.8 ± 1.2	2.6 ± 0.6		2.6 ± 1.0	2.4 ± 0.8	2.1 ± 0.6	1.3 ± 0.8
22:5‖					0.8 ± 0.3				1.0 ± 0.1
22:6	9.1 ± 1.8	10.9 ± 3.0	9.7 ± 2.6	14.9 ± 1.8	18.4 ± 2.6	9.6 ± 2.6	10.9 ± 1.8	8.7 ± 2.2	16.6 ± 3.8
24:0	2.9 ± 0.4	2.9 ± 1.4	3.3 ± 0.4	3.9 ± 0.4	2.2 ± 1.0	3.2 ± 1.2	3.6 ± 1.0	3.4 ± 0.6	1.3 ± 0.8
mg ester/mg nitrogen in mitochondria		1.4 ± 0.3	1.4 ± 0.4	1.3 ± 0.3	1.2 ± 0.2		1.4 ± 0.5	1.3 ± 0.1	1.2 ± 0.2

* Percentage of total area of gas-liquid chromatographic elution diagram.
† Standard deviation (5 samples).
‡ Saturated aliphatic aldehydes.
§ $\Delta^{5,8,11,14,17}$ 20:5 from cod liver oil.
‖ $\Delta^{7,10,13,16,19}$ 22:5 from cod liver oil.

same animal showed a marginal deficiency by this criterion.

The fatty acid compositions of tissues including brain have been found to depend upon the composition of the dietary fat. A logical question follows: What concentrations of various polyunsaturated acids in different tissues suffice to prevent an essential fatty acid deficiency, and how may a marginal deficiency be recognized?

Polyunsaturated fatty acids fall into three groups depending on whether the first double bond, numbered from the terminal methyl group, is in the 3-, 6-, or 9-position. Compounds having full essential fatty acid activity have the first double bond in the 6-position (30). Linolenic acid (18:3) does not fulfill all the functions of an essential fatty acid (31). Appearance in the tissue of fatty acids belonging to the other groups, particularly eicosatrienoic acid (20:3), has been considered as a symptom of essential fatty acid deficiency (15) in which the body desaturates nonessential fatty acids in a futile attempt to meet its requirements.

When the linoleic acid (18:2) content of liver mitochondrial lipids fell below approximately 10% in animals on the 0.2% corn oil or 15% coconut oil diets, nonessential polyunsaturated fatty acids (20:3) accumulated. This occurred despite the presence of 9% to 17% arachidonic acid (20:4) in the same tissue. These nonessential polyunsaturated fatty acids were not elevated in the liver mitochondrial lipids of the weanlings or the corn oil groups (14% and 20% to 21% linoleic acid [18:2], respectively). In the groups receiving cod liver oil, the linoleic acid (18:2) concentration in the fatty acids of the liver mitochondrial lipids fell to 5% to 6% without production of eicosatrienoic acids (20:3), possibly as a result of the presence of 9% to 15% of nonessential pentaenoic acids ($\Delta^{5,8,11,14,17}$ 20:5 and $\Delta^{7,10,13,16,19}$ 22:5) derived from the diet. Arachidonic acid (20:4) was also very low (5% to 6%) in this group. The elevation of docosahexaenoic acid (22:6) concentration probably arises largely from incorporation of a dietary fatty acid present in cod liver oil with the same retention time as the docosahexaenoic acid (22:6) usually present in mammalian tissue. Since this fatty acid from cod liver oil probably has the 4, 7, 10, 13, 16, 19 double bond sequence (24), and is therefore a member of the linolenic acid (18:3) family, full essential fatty acid activity is precluded (30).

In the lipids of the erythrocyte, concentrations of 6% linoleic acid (18:2) and 11% arachidonic acid (20:4) appeared to meet adequately the essential fatty acid requirements of the weanling, while concentrations

of 6% to 8% linoleic acid (18:2) and 23% arachidonic acid (20:4) did not quite adequately meet the requirements for the 24-week-old animals, as judged by the appearance of nonessential polyunsaturated fatty acids. The erythrocytes from the 15% corn oil group contained 13% linoleic acid (18:2), and 23% arachidonic acid (20:4). The feeding of the nonessential polyunsaturated fatty acids of cod liver oil produced in erythrocytes the same fatty acid patterns as those described above for liver mitochondrial lipids.

Brain mitochondrial lipids contain very little linoleic acid (18:2). However, eicosatrienoic acids (20:3) did not accumulate when marginal essential fatty acid deficiency was evidenced by other tissues. The data suggest that linoleic acid (18:2) and arachidonic acid (20:4) should probably account for 1% to 2% and 10% to 12%, respectively, of the brain mitochondrial fatty acids of rats on an adequate diet, and that levels lower than these may be considered representative of a deficiency. In weanlings, the brain mitochondrial lipids contained little linoleic acid (18:2); however, when 0.2% corn oil or 15% coconut oil diets were fed, there were no appreciable accumulations of eicosatrienoic acids (20:3). When the cod liver oil diet was fed, arachidonic acid (20:4) was considerably decreased, and in all groups docosahexaenoic acid (22:6) constituted the major polyenoic acid.

The data presented above suggest that the relative content of essential fatty acids in a given tissue may be an index of the nutritional status of the animal. Normal requirements for these acids in the tissues studied are higher than anticipated. These needs cannot be judged in terms of their collective total, but rather in terms of requirements for the individual fatty acids for a given tissue (2, 3). For example, the erythrocyte and the mitochondria of the brain and liver contain approximately 25% to 35% of essential fatty acids. In the case of the erythrocyte, arachidonic acid (20:4) is most prevalent; in the brain mitochondria, docosahexaenoic acid (22:6) is a principal constituent; and in the liver, arachidonic acid (20:4) and linoleic acid (18:2) are present in similar high concentrations. It should be noted that while the first measurable indication of a marginal deficiency is the accumulation of nonessential polyunsaturated fatty acids, particularly the trienoic acids (20:3), this test fails if the diet supplies even more highly unsaturated nonessential fatty acids such as those in cod liver oil. The trienoic acids (20:3) do not appear in this case.

Although the appearance of a trienoic acid ($\Delta^{5,18,11}$ 20:3) has been recognized as the first sign of impending deficiency (14, 15), the existence of two isomeric trienoic acids (20:3), appears to have been overlooked in studies utilizing alkali isomerization as the sole analytical method. This represents a serious drawback to the use of the alkali isomerization technique in studies of essential fatty acid deficiency. In the liver mitochondrial lipids, where deficiency was noted when linoleic acid (18:2) constituted less than approximately 10% of the fatty acids, the user of the alkali isomerization technique would measure approximately 6% dienoic acid, and base his estimate of nonessential polyunsaturated fatty acids on the detection of 7% trienoic acids (20:3) without realizing two fatty acids were making up the observed triene content.

In recent years there has been growing awareness of the biochemical importance of the fatty acid composition of the phospholipids. This has been especially pertinent with respect to the unsaturated fatty acids in the diglycerides, which are the preferred substrates for phosphatidic acid synthesis (32, 33). Furthermore, where phosphate exchange in phosphatidic acids has been studied in relation to cholinergic agents, the fatty acid composition of the phosphatidic acid may be a determinant in the rate of active transport across a membrane (34). Collins (35) has shown that rat liver lecithins which contained the most highly unsaturated fatty acids were most highly labeled when P^{32} inorganic phosphate was given *in vivo*. A large difference in specific activity was noted between the most highly unsaturated lecithins.

When essential fatty acids are replaced by nonessential polyunsaturated fatty acids, well-known symptoms of essential fatty acid deficiency become apparent. Conversely, if there is a high intake of polyunsaturated fatty acids, inadequately protected against *in vivo* autoxidation, other pathological manifestations may appear (2, 36).

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REFERENCES

1. Holman, R. T., W. O. Lundberg and T. Malkin. In *Progress in the Chemistry of Fats and Other Lipids*, edited by H. J. Deuel, Jr., New York, Pergamon Press, 1954, vol. 2, p. 99.
2. Century, B., M. K. Horwitt and P. Bailey. *A.M.A. Arch. Gen. Psychiat.* **1:** 420, 1959.
3. Century, B., and M. K. Horwitt. *Proc. Soc. Exptl. Biol. Med.* **102:** 375, 1959.
4. Sugai, M., M. Inoue, H. Tsuchiyama, and F. A. Kummerow. *Federation Proc.* **19:** 421, 1960.

5. Kokatnur, M., M. Uchiyama, S. Okui, H. Tsuchiyama, F. A. Kummerow, and H. M. Scott. *Federation Proc.* **19:** 421, 1960.
6. Horwitt, M. K., and P. Bailey. *Arch. Neurol.* **1:** 312, 1959.
7. Century, B., and M. K. Horwitt. *J. Nutrition* **72:** 357, 1960.
8. Horwitt, M. K., C. C. Harvey and B. J. Meyer. *Federation Proc.* **17:** 245, 1958.
9. Horwitt, M. K., C. C. Harvey, G. D. Duncan, and W. C. Wilson. *Am. J. Clin. Nutrition* **4:** 408, 1956.
10. Horwitt, M. K., C. C. Harvey and B. Century. *Science* **130:** 917, 1959.
11. Pihl, A., K. Bloch and H. S. Anker. *J. Biol. Chem.* **183:** 441, 1950.
12. Burr, G. O., and R. H. Barnes. *Physiol. Revs.* **23:** 256, 1948.
13. Hegsted, D. M., C. Whyman, A. Gotsis, and S. A. Andrus. *Am. J. Clin. Nutrition* **8:** 209, 1960.
14. Dam, H., and P. F. Engel. *Acta Physiol. Scand.* **42:** 28, 1958.
15. Holman, R. T. *J. Nutrition* **70:** 405, 1960.
16. Morris, S. G., R. W. Riemenschneider and J. D. Evans. *Arch. Biochem. Biophys.* **78:** 138, 1958.
17. Brody, T. M., and J. A. Bain. *J. Biol. Chem.* **195:** 685, 1952.
18. Stoffel, W., F. Chu and E. H. Ahrens, Jr. *Anal. Chem.* **31:** 307, 1959.
19. Clayton, M. M., P. A. Adams, G. B. Mahoney, S. W. Randall, and E. T. Schwartz. *Clin. Chem.* **5:** 426, 1959.
20. Hiller, A. *Practical Clinical Chemistry*, Springfield, Ill., Charles C Thomas, 1953, p. 106.
21. Ahrens, E. H., Jr., J. Hirsch, W. Insull, Jr., M. L. Peterson, W. Stoffel, J. W. Farquhar, T. Miller, and H. Thomasson. *Lancet* **1:** 115, 1959.
22. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr. *Nutrition Revs.* **17:** Supplement, No. 18, Part II, 1959.
23. Klenk, E., and D. Eberhagen. *Z. physiol. Chem., Hoppe-Seyler's* **307:** 42, 1957.
24. Klenk, E. *First International Conference on Biochemical Problems of Lipids*, Brussels, 1953, p. 33.
25. Mead, J. F., and W. H. Slaton, Jr. *J. Biol. Chem.* **219:** 705, 1956.
26. Fulco, A. J., and J. F. Mead. *J. Biol. Chem.* **234:** 1411, 1959.
27. Holman, R. T., and H. Hayes. *Anal. Chem.* **30:** 1422, 1958.
28. Spiro, M. J., and J. M. McKibbin. *J. Biol. Chem.* **219:** 643, 1956.
29. Mead, J. F., and D. L. Fillerup. *J. Biol. Chem.* **227:** 1009, 1957.
30. Thomasson, H. J. *First International Conference on Biochemical Problems of Lipids*, Brussels, 1953, p. 212.
31. Holman, R. T., and E. Aaes-Jorgensen. *Proc. Soc. Exptl. Biol. Med.* **93:** 175, 1956.
32. Hokin, M. R., and L. E. Hokin. *J. Biol. Chem.* **234:** 1381, 1959.
33. Weiss, S. B., E. P. Kennedy and J. Y. Kiyasu. *J. Biol. Chem.* **235:** 40, 1960.
34. Hokin, L. E., and M. R. Hokin. *J. Biol. Chem.* **233:** 800, 1958.
35. Collins, F. D. *Nature* **186:** 366, 1960.
36. Horwitt, M. K. *Am. J. Clin. Nutrition* **8:** 451, 1960.

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Notes on Methodology

Cholesterol measurement in normal and lipemic sera: elimination of an extraneous chromogen

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» Unusually high and variable cholesterol values were obtained in an attempt to apply the method of Zlatkis *et al.* (1) to normal and lipemic sera (Table 1). Further investigation demonstrated that the variability could be reduced by extraction and saponification of the lipids prior to color development; the values so obtained were in agreement with those found using the method of Sperry and Webb (2). The resulting procedure, incorporating these findings, permits the rapid determination of cholesterol in both tissues and sera at macro and micro levels.

Procedure. The lipids are coprecipitated with the proteins from a sample containing from 0.2 to 10 mg of cholesterol by the addition of trichloroacetic acid to a final concentration of from 5% to 10%. After centrifuging and discarding the supernatant liquid, the total lipids are extracted from the residue with 2 ml of 0.1 N potassium acetate in absolute alcohol. The extraction is repeated twice with 2 ml of absolute alcohol each time, and the three extracts are combined. Saponification is carried out by adding 0.2 ml of 33% (w/w) KOH to the combined alcohol extracts and incubating at 40° for 1 hour. The nonsaponifiable lipids are subsequently extracted into 10 ml of high boiling petroleum ether, using the technique described by Abell *et al.* (3), and a suitable aliquot of the petroleum ether extract is evaporated to dryness. A standard and a blank are carried through the saponification, petroleum ether extraction, and drying steps. The dried residues are dissolved in glacial acetic acid, and color development and measurement are carried out, using the ferric chloride reagent and the technique described by Zlatkis *et al.* (1).

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TABLE 1. EFFECT OF EXTRACTION AND SAPONIFICATION ON CHOLESTEROL VALUES FOR HUMAN SERA

Sample No.	Without Extraction*	With Extraction and Saponification		
		Spectrophotometric†	Fluorometric†	Sperry and Webb‡
	mg/100 ml	*mg/100 ml*	*mg/100 ml*	*mg/100 ml*
1	249	152	146	146
2	268	162	158	159
3	321	198	190	200
4	259	152	148	156
5	273	166	163	164
6	285	178	171	189
7	270	172	166	175

* Direct method of Zlatkis *et al.* (1).
† Sample preparation and measurement as described in the text.
‡ See Ref. 2.

By reducing the volume tenfold, the same sample preparation technique may be carried out at a micro level, and the cholesterol measured with a Farrand Model A fluorometer (4). Because of the increased sensitivity of the fluorometric measurement, the original sample need only contain from 2 to 100 μg of cholesterol. The reagent of Albers and Lowry (4) and their method of addition have been modified as follows: Acetic anhydride, 1,1,2-trichloroethane, and sulfuric acid are mixed in the proper proportion immediately before use. One milliliter of the reagent is added to the dried residues with immediate and thorough mixing. This modification results in an approximately twofold increase in the galvanometer deflection for the same amount of cholesterol, and requires only one manipulation instead of two (Fig. 1). Lang-Levy constriction pipettes (5) are used for all additions and transfers in the micromethod and a "buzzer" (6) is used for mixing.

The procedures employed in the sample preparation

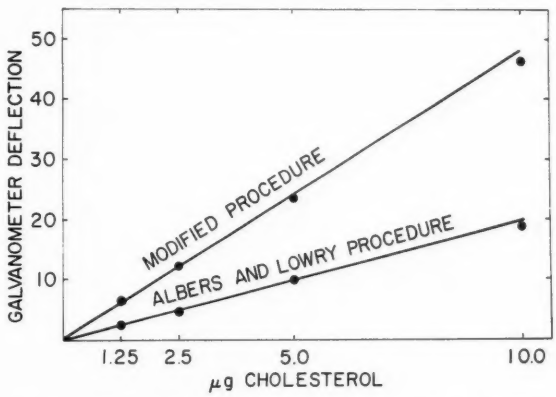

FIG. 1. Increased sensitivity using proposed reagent and method of addition.

have been demonstrated to be quantitative by various authors and by the data included herein. Tourtellotte *et al.* (7), Robins *et al.* (8), and Zilversmit and Davis (9) have demonstrated that lipids are quantitatively coprecipitated with proteins by trichloroacetic acid at a final concentration of from 5% to 10%. The use of this procedure makes possible the precipitation of lipids from various types of samples. Robins *et al.* (8) and Albers and Lowry (4) have shown that lipids are quantitatively extracted from the protein precipitate by alcoholic potassium acetate and absolute alcohol, as described. Abell *et al.* (3) have shown that cholesterol is quantitatively extracted into petroleum ether from an equal volume of 50% alcohol.

Table 2 shows the apparent cholesterol level of pooled rat sera when measured directly, and the level in various fractions prepared according to the method described in the text. The petroleum ether extract yields a value which is in good agreement with the value obtained by the method of Sperry and Webb (2). In addition, C^{14}-cholesterol added to the original samples is quantitatively recovered in the petroleum ether extract, indicating that the decrease in the cholesterol level was not due to loss of the sterol but rather to the removal of interfering substances prior to measurement. This is confirmed by the spectral data (Fig. 2). The spectrum of the petroleum ether fraction closely parallels that of cholesterol and produces a lower absorbency at 560 mμ than the directly measured serum or the unsaponified alcohol extract. Equivalent amounts of both the trichloroacetic acid precipitate and the saponifiable material contribute to the absorbency of 560 mμ, and hence produce the high value for cholesterol when serum is measured directly.

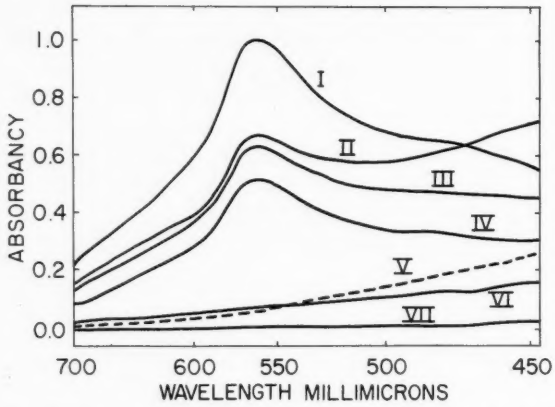

FIG. 2. Spectrum of cholesterol, serum, and fractions obtained during sample preparation. Color development and measurement as described in Ref. 1. *I*, cholesterol; *II*, serum; *III*, unsaponified ethanol extract; *IV*, petroleum ether extract; *V*, redissolved trichloroacetic acid precipitate after extraction of lipids; *VI*, saponified ethanol extract after removal of nonsaponifiable material; and *VII*, reagent blank.

The exact nature of the interfering material has not been established, but the need for its removal prior to measurement is evident from the preceding experiments. The method of Sperry and Webb (2) removes extraneous material by digitonin precipitation of the sterol, which requires approximately 13 hours for completion. The present procedure allows analysis to be completed in a single day, and may be applied to tissues as well as sera on either a macro or micro level.

TABLE 2. CHOLESTEROL VALUES OF VARIOUS FRACTIONS OF RAT SERUM AND RECOVERY OF ADDED C^{14}-CHOLESTEROL

Sample	A	B	A	B
Fraction	Cholesterol			
	mg/100 ml		*cpm* × 10^{-3}*	
Serum†	137	67	906	907
Unsaponified ethanol extract‡	128	65	924	924
Petroleum ether extract‡	102	57	917	912
Digitonin precipitate§	98	51		

* C^{14}-cholesterol was added to the serum sample and an aliquot was removed for direct determination of cholesterol and counting. The remainder of the sample was fractionated as described in the text and aliquots of the fractions were removed for cholesterol measurement and counting. Counting was done in a liquid scintillation counter using the solvent described by Werbin *et al.* (10).

† Analysis by direct method (1).

‡ Sample preparation as described in text. Color development and measurement as described in Ref. 1.

§ Analysis by the method of Sperry and Webb (2).

REFERENCES

1. Zlatkis, A., B. Zak and A. J. Boyle. *J. Lab. Clin. Med.* **41:** 486, 1953.
2. Sperry, W. M., and M. Webb. *J. Biol. Chem.* **187:** 97, 1950.
3. Abell, L. L., B. B. Levy, B. B. Brodie, and F. E. Kendall. *J. Biol. Chem.* **195:** 357, 1952.
4. Albers, R. W., and O. H. Lowry. *Anal. Chem.* **27:** 1829, 1955.
5. Lowry, O. H., N. R. Roberts, K. Y. Leiner, Mei-Ling Wu, and A. L. Farr. *J. Biol. Chem.* **207:** 1, 1954.
6. Bessey, O. A., O. H. Lowry, M. J. Brock, and J. A. Lopez. *J. Biol. Chem.* **166:** 177, 1946.
7. Tourtellotte, W. W., A. J. Vander, B. A. Skrentny, and R. N. De Jong. *J. Lab. Clin. Med.* **52:** 480, 1958.
8. Robins, E., O. H. Lowry, K. M. Eydt, and R. E. McCaman. *J. Biol. Chem.* **220:** 661, 1956.
9. Zilversmit, D. B., and A. K. Davis. *J. Lab. Clin. Med.* **35:** 155, 1950.
10. Werbin, H., I. L. Chaikoff and M. R. Imada. *Proc. Soc. Exptl. Biol. Med.* **102:** 8, 1959.

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New Methods

» New Methods is a listing of pertinent references to analytical lipid methods published recently. It is compiled by H. A. I. Newman and Norman S. Radin. (References opened with No. 1 in the January, 1961, issue of the JOURNAL OF LIPID RESEARCH.)

364. SEPARATION OF URINARY 17-KETOSTEROIDS BY GAS CHROMATOGRAPHY. Haahti, E. O. A., W. J. A. VandenHeuvel and E. C. Horning. *Anal. Biochem.* **2:** 182, 1961.

365. THE DETERMINATION OF CITRONELLOL IN ADMIXTURE WITH GERANIOL: FURTHER STUDIES OF FORMYLATION REACTIONS BY GAS-LIQUID CHROMATOGRAPHY. Holness, D. *Analyst* **86:** 231, 1961.

366. THE QUANTITATIVE DETERMINATION OF GLYCEROL, SERINE, ETHANOLAMINE, AND FATTY ACIDS IN LIPIDS BY GAS-LIQUID CHROMATOGRAPHY. Horrocks, L. A. *Diss. Abstr.* **21:** Mic. 2876, 1961.

367. ANALYSIS OF GAS-LIQUID CHROMATOGRAMS BY A PUNCHED CARD TECHNIQUE. Tandy, R. K., F. T. Lindgren, W. H. Martin, and R. D. Wills. *Anal. Chem.* **33:** 665, 1961.

368. A NEW LIQUID PHASE FOR GAS CHROMATOGRAPHIC SEPARATIONS OF STEROIDS. VandenHeuvel, W. J. A., E. O. A. Haahti and E. C. Horning. *J. Am. Chem. Soc.* **83:** 1513, 1961.

369. DETERMINATION OF TOTAL LIPID IN THE CEREBROSPINAL FLUID BY PAPER CHROMATOGRAPHY. Curtis, W. C., and J. H. Seipel. *J. Neurochem.* **6:** 318, 1961.

370. ANALYSES OF LIPIDS AND OXIDATION PRODUCTS BY PARTITION CHROMATOGRAPHY. DIMERIC AND POLYMERIC PRODUCTS. Frankel, E. N., C. D. Evans, H. A. Moser, D. G. McConnell, and J. C. Cowan. *J. Am. Oil Chemists' Soc.* **38:** 130, 1961.

371. A NOTE ON A RAPID METHOD FOR THE DETERMINATION OF LIPIDS IN BREWING ADJUNCT CEREALS. Haas, G. J., and A. I. Fleischman. *Cereal Chem.* **38:** 198, 1961.

372. FRACTIONATION OF COMPOUND LIPIDS BY DIALYSATION IN AN ORGANIC SOLVENT AGAINST AN ORGANIC SOLVENT. Hakomori, S., and K. Takeda. *Nature* **190:** 265, 1961.

373. AUTOXIDATION OF MILK LIPIDS. II. THE RELATIONSHIP OF SENSORY TO CHEMICAL METHODS FOR MEASURING THE OXIDIZED FLAVOR OF MILK FATS. Lillard, D. A., and E. A. Day. *J. Dairy Sci.* **44:** 623, 1961.

374. A MICROMETHOD FOR FRACTIONATION OF LIPIDS BY SILICIC ACID CHROMATOGRAPHY. Lis, E. W., J. Tinoco and R. Okey. *Anal. Biochem.* **2:** 100, 1961.

375. A MODIFIED RECORDING OXYGEN ABSORPTION APPARATUS FOR THE DETERMINATION OF OXIDATIVE STABILITY OF LIPID MATERIAL. Martin, H. F. *Chem. & Ind. (London)*, 364 (1961).

376. THE EFFECT OF RELATIVE CONCENTRATIONS ON THE EFFICIENCY OF SEPARATION OF POLAR AND NONPOLAR LIPIDS BY ALUMINA COLUMN CHROMATOGRAPHY. Sims, R. P. A., and J. C. Mes. *J. Am. Oil Chemists' Soc.* **38:** 229, 1961.

377. CHROMATOGRAPHY OF LIPIDS ON SILICIC ACID: INFRARED SPECTROPHOTOMETRIC ELUTION CURVES. Wren, J. J., and P. M. Lenthen. *J. Chromatog.* **5:** 370, 1961.

378. DETERMINATION OF STEROIDS BY PAPER STRIP ELUTION CHROMATOGRAPHY. Abelson, D. M., P. K. Bondy and J. Piskorski. *J. Chromatog.* **5:** 332, 1961.

379. REVERSED PHASE PARTITION CHROMATOGRAPHY OF STEROIDS. Carr, H. E., Jr., and W. J. Reddy. *Anal. Biochem.* **2:** 152. 1961.

380. Determinazione di Steroidi in Estratti di Corteccia Surrenale Mediante Cromatografia su Carta. Cavina, G., E. Cingolani and L. Tentori. *Il Farmaco* **16**: 3, 1961.

381. The Estimation of Urinary Metabolites of Administered Estradiol-17β-16-C^{14}. Engel, L. L., C. B. Cameron, A. Stoffyn, J. A. Alexander, O. Klein, and N. D. Trofimow. *Anal. Biochem.* **2**: 114, 1961.

382. A Method for the Analysis of Urinary 17-Hydroxycorticosteroids. Few, J. D. *J. Endocrinol.* **22**: 31, 1961.

383. Studies on High Voltage Paperelectrophoreses on Adrenocortical Steroids and Its Related Compounds (I). Iso, T. *Seikagaku* **33**: 103, 1961.

384. A Simple Method for the Determination of Urinary Adrenocortical Steroids (α-Ketols). Kingsley, G. R., and G. Getchell. *Anal. Biochem.* **2**: 1, 1961.

385. Separation and Determination of the Preparations of Mixed Steroids. II. Determination of Methyltestosterone and Ethisterone from the Tablet Containing Methyltestosterone, Ethisterone, and Ethynylestradiol. Kitazawa, T., M. Naito, F. Yoshida, and M. Maruyama. *Yakugaku Zasshi* **81**: 496, 1961.

386. Extension of the Porter-Silber Reaction to 17-Deoxy-Alpha-Ketolic Steroids. Lewbart, M. L., and V. R. Mattox. *Anal. Chem.* **33**: 559, 1961.

387. La Determininazione Colorimetrica e Cromatografica Degli Estrogeni Urinari. Marescotti, V., L. M. Savi and C. Savi. *Boll. soc. ital. biol. sper.* **37**: 363, 1961.

388. Rapid Determination of 17α-Oxy-11-Desoxy-Corticosterone in the Course of Biological Fermentative Oxidations. Mizsei, A., and A. Szabó. *Biochem. Microbiolog. Technol. Eng.* **3**: 21, 1961.

389. Der Mechanismus der Zimmermann-Reaktion (1935–1960). Darstellung des 16-(aci-2,4-Dinitro-Phenyl)-Androsterons. Neunhoeffer, O., K. Thewalt and W. Zimmermann. *Z. physiol. Chem., Hoppe-Seyler's* **323**: 116, 1961.

390. A Study of the Colorimetric Estimation of Oestradiol-17β, Oestradiol-17α, Oestrone, Oestriol, and 16-Epiostriol by the Kober Reaction. Nocke, W. *Biochem. J.* **78**: 593, 1961.

391. Pregnanediol. Methods, Limitations, and Applications. Ober, W. B., and G. A. Kaiser. *Am. J. Clin. Pathol.* **35**: 297, 1961.

392. Quantitative Bestimmung von Pregnandiol. Oertel, G. W., and E. Kaiser. *Klin. Wochschr.* **39**: 492, 1961.

393. Chromatographie d'Adsorption sur Papier des Acétates de Stéroides peu Polaires. Pasqualini, J. R., and M. F. Jayle. *J. Chromatog.* **5**: 336, 1961.

394. The Determination of Small Quantities of Urinary Oestrone, Oestradiol-17β, and Oestriol Using Ittrich's Extraction Method. Salokangas, R. A. A., and R. D. Bulbrook. *J. Endocrinol.* **22**: 47, 1961.

395. An Appraisal of Laboratory Determinations of Estrogens. Sommers, S. C., and J. B. Verendia. *Am. J. Clin. Pathol.* **35**: 319, 1961.

396. A Simple Paper Chromatographic Purification Method of Extracts for Corticosteroid Determination in Rat Experiments. Weisz, P., and E. Gláz. *Med. Exptl.* **3**: 264, 1960.

397. A Quantitative Chromatographic Assay of Bile Acids in the Bile and Blood. Karbach, J. I. *Biokhimiya* (USSR) **26**: 305, 1961.

398. Determination Colorimetrique Simple et Rapide du Cholesterol Libre en Presence de ses Esters d'Acides Gras. Assous, É., and M. Girard. *Compt. rend.* **252**: 2462, 1961.

399. Turbidimetric Determination of Total Serum Cholesterol. Kingsley, G. R., and O. Robnett. *Anal. Chem.* **33**: 561, 1961.

400. Analyses of Lipids and Oxidation Products by Partition Chromatography, Fatty Acid Hydroperoxides. Frankel, E. N., C. D. Evans, D. G. McConnell, and E. P. Jones. *J. Am. Oil Chemists' Soc.* **38**: 134, 1961.

401. The Infrared Dichroism of Methyl Stearate and Elaidic Acid. Kawano, M. *J. Chem. Soc. Japan* **82**: 427, 1961.

402. The Infrared Dichroism of n-Fatty Acids. Kawano, M. *J. Chem. Soc. Japan* **82**: 427, 1961.

403. Determination of Multiple Bonds by Reversed Phase Paper Chromatography. McHale, D., J. Green and S. Marcinkiewicz. *Chem. & Ind.* (*London*), 555 (1961).

404. Enzymatische Bestimmung von Polyensäuren im Serum. Süllmann, H. *Klin. Wochschr.* **39**: 386, 1961.

405. Identification of Hydroxamic Acids by Gas Chromatography of Isocyanate Derivatives. Vagelos, P. R., W. J. A. VandenHeuvel and M. G. Horning. *Anal. Biochem.* **2**: 50, 1961.

406. New Solvent System for Separation of Fatty Acids C_{10}–C_{18} by Countercurrent Distribution. Will, F., III. *Anal. Chem.* **33**: 647, 1961.

407. A New Method for the Detection of Diglycerides on a Microscale. Clark, B. F. C. *J. Chromatog.* **5**: 368, 1961.

408. Chromatography of Lipids and Its Biochemical Applications. Part IV. A Simple Method for Analysis of Triglycerides by Paper Chromatography. Hirayama, O., and Y. Inoue. *Nippon Nogei-kagaku Kaishi* **35**: 367, 1961.

409. Chromatography of Lipids and Its Biochemical Applications. Part V. Paper Chromatographic Analysis of Fatty Acid, Mono-, Di-, and Triglyceride Mixture. Hirayama, O., and Y. Inoue. *Nippon Nogei-kagaku Kaishi* **35**: 372, 1961.

410. Chromatography of Lipids and Its Biochemical Applications. Part VI. Chromatographic Separations of Mixed Triglycerides on Silicic Acid Column. Hirayama, O. *Nippon Nogei-kagaku Kaishi* **35**: 437, 1961.

411. Chromatography of Lipids and Its Biochemical Applications. Part VII. Paper Chromatographic Analyses of Component Glycerides in Soybean Oil and Stillingia Oil. Hirayama, O. *Nippon Nogei-kagaku Kaishi* **35**: 441, 1961.

412. Glyceride Structure of Vegetable Oils by Countercurrent Distribution. VI. Corn Oil. Scholfield, C. R., J. Nowakowska and H. J. Dutton. *J. Am. Oil Chemists' Soc.* **38**: 175, 1961.

413. Separation of Some Glycerides of Cocoa Butter by Paper Chromatography. Steiner, E. H., and A. R. Bonar. *J. Sci. Food Agr.* **12**: 247, 1961.

414. Differentiation of 1- and 2-Monoglycerides by Near-Infrared Absorption Spectroscopy. Susi, H., S. G. Morris and W. E. Scott. *J. Am. Oil Chemists' Soc.* **38**: 199, 1961.

415. Spectrophotometric Determination of Submicrogram Quantities of Orthophosphate in Natural Waters. Sugawara, K., and S. Kanamori. *Bull. Chem. Soc. Japan* **34**: 258, 1961.

416. Dünnschictchromatographie von Phosphatiden und Glykolipiden. Mit 4 Textabbildungen. Wagner, H., L. Hörhammer and P. Wolff. *Biochem. Z.* **334**: 175, 1961.

417. The Quantitative Separation and Estimation of Glycosphingosides in Nervous Tissue, with Especial Reference to Gangliosides. Payne, S. N., and B. S. Platt. *J. Neurochem.* **7**: 5, 1961.

418. Essential Oils and Related Products. Guenther, E., K. Kulka and J. A. Rogers. *Anal. Chem.* **33**: 37R, 1961.

419. Analyse des Huiles Essentielles par Chromatographie sur Papier et sur Plaques. Application à Quelques Essences Officinales. Paris, R., and M. Gordon. *Ann. pharm. franç.* **19**: 86, 1961.

420. Vitamin D Assay in Animal Tissues. Bukin, V. N., and I. N. Garkina. *Biokhimiya* (USSR) **26**: 40, 1961.

421. Die Analyse von Tocopherolgemischen mit Hilfe der Dünnschichtchromatographie. Seher, A. *Mikrochim. Acta* **2**: 308, 1961.

422. A Simple Method for Quantitation of Serum Beta-Lipoproteins by Means of the Immunocrit. Heiskell, C. L., R. T. Fisk, W. H. Florsheim, A. Tachi, J. R. Goodman, and C. M. Carpenter. *Am. J. Clin. Pathol.* **35**: 222, 1961.

423. Measurement of Lecithinase A in Serum and Other Body Fluids. Zieve, L., and W. C. Vogel. *J. Lab. Clin. Med.* **57**: 586, 1961.

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Unsaturated Fatty Acids to Be Distributed by National Institutes of Health Lipid Program

A co-operative program sponsored by the Division of Research Grants and the National Heart Institute, National Institutes of Health, will soon distribute ampoules containing 100 mg of each of 8 unsaturated fatty acid methyl esters. Available in 99% purity will be esters of docosa-13-enoate (erucate), eicosa-11-enoate, octa-12-enoate, and octa-11-enoate; samples of 95% or greater purity will include eicosa-5,8,11,14,17-pentaenoate, docosa-4,7,10,13,16,19-hexaenoate, tetracosa-15-enoate (nervonate), and octadeca-6-enoate (petroselenate).

Qualified investigators desiring sets of these samples should describe briefly the research program for which they are to be used. The Committee advising this activity will attempt to fill all deserving requests, although aiming also to assure broad distribution to areas where they may be needed. Letters should be addressed in duplicate to Dr. William H. Goldwater, Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.

J. Lipid Research, October, 1961
Volume 2, Number 4

New Methods Index

Vol. 2, Nos. 1–4

Compiled by H. A. I. Newman and John W. Farquhar

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INSTRUCTIONS TO AUTHORS

Scope: The JOURNAL OF LIPID RESEARCH, a quarterly, will publish original articles dealing with the chemistry, biochemistry, enzymology, histochemistry, and physiology of the lipids. The editors will favorably consider significant contributions in the field of lipid methodology and will encourage publication of sufficient details so that the methods can be reproduced. Clinical observations and nutritional data will be welcomed if they offer contributions in the above fields. In addition to original articles, the JOURNAL will contain review articles and a section, "Notes on Methodology," dealing primarily with minor improvements in existing methodology. Notes on Methodology will not generally require a summary or subdivision into sections. A listing of new lipid methods published in other journals will also be provided. Although articles will be published in English only, it is hoped that the JOURNAL will become a vehicle for international communication.

Preparation of manuscripts: All manuscripts, including tables and figures, should be submitted in duplicate. Manuscripts should be typewritten, double-spaced, with minimum page margins of one inch. The first page should be a title page, consisting of: title; author(s); institution from which the paper is submitted, including the complete postal address(es) for mailing of proofs and reprint requests; and an abbreviated title for a running head (not to exceed 60 characters, counting the spaces between words). A notation should be made when the manuscript is intended for the Notes on Methodology section. Footnotes on the title page and those to tables should be indicated by non-numerical symbols (asterisk, dagger, double dagger, etc.), but in the text should be numbered consecutively and typed on a separate sheet.

Manuscripts should be written in clear, grammatical English. Unusual abbreviations should be used as little as possible, and must always be initially defined. Each article should be preceded by a Summary, not to exceed 200 words. Whenever possible, Methods, Results, and Discussion should be set forth in separate sections with appropriate headings. These headings should be in capital letters, centered on the page; subheadings should be in lower case and underlined for italicizing in print. All drawings and illustrations should be submitted in the form of glossy prints. Each should be identified on the back by figure number and author's name, using a *soft pencil* to avoid marring the print.

Tables should be typed on separate sheets and numbered with Arabic figures. All tables should be self-explanatory. Draw graphs and diagrams in *black* ink.

For the terminology of lipid enzymes the JOURNAL will follow the recommendations published in "Nomenclature of enzymes of fatty acid metabolism" in *Biochemical Problems of Lipids*, edited by G. Popják and E. Le Breton, London, Butterworths Scientific Publications, 1956, p. 246.

For the terminology of serum lipoproteins the JOURNAL will follow the recommendations published in *Circulation Research* **4**: 129, 1956.

The following are the accepted abbreviations for units of measurement:

Units of Mass:	
kilogram	kg
gram	g
milligram	mg
microgram	μg
millimole	mmole (not mM)
micromole	μmole (not μM)
Units of Concentration:	
molar (mole per liter)	M
millimolar	mM
micromolar	μM
Units of Length, Area, Volume, etc.:	
meter	m
centimeter	cm
millimeter	mm
millimicron	mμ
square centimeter	cm^2
liter	liter
milliliter	ml
cubic centimeter	cc or cm^3
microliter	μl
counts per minute	cpm
counts per second	cps
millicurie	mc
microcurie	μc
temperature (Centigrade only)	°

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